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A ligase-mediated gene detection technique.

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An assay for the presence of given DNA sequences has been developed, based on the ability of two oligonucleotides to anneal immediately adjacent to each other on a complementary target DNA molecule. The two oligonucleotides are then joined covalently by the action of a DNA ligase, provided that the nucleotides at the junction are correctly base-paired. Thus single nucleotide substitutions can be distinguished. This strategy permits the rapid and standardized identification of single-copy gene sequences in genomic DNA.

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Genetics

Genetic disease detection and DNA amplification using cloned thermostable ligase

(β-globin gene/ligase chain reaction/sickle-cell allele/single-base mutation)

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ABSTRACT **Polymerase chain reaction, using thermostable DNA polymerase, has revolutionized DNA diagnostics. Another thermostable enzyme, DNA ligase, is harnessed in the assay reported here that both amplifies DNA and discriminates a single-base substitution. This cloned enzyme specifically links two adjacent oligonucleotides when hybridized at 65°C to a complementary target only when the nucleotides are perfectly base-paired at the junction. Oligonucleotide products are exponentially amplified by thermal cycling of the ligation reaction in the presence of a second set of adjacent oligonucleotides, complementary to the first set and the target. A single-base mismatch prevents ligation/amplification and is thus distinguished. This method was exploited to detect 200 target molecules as well as to discriminate between normal β^A- and sickle β^S-globin genotypes from 10-μl blood samples.**

DNA diagnostics uses the tools of molecular biology to identify nucleotide substitutions, deletions, or insertions in genes of medical interest (1). A reliable DNA diagnostics method will require faithful amplification of target sequences, accurate single-base discrimination, low background, and, ultimately, complete automation. The initial target nucleic acid amplification may be accomplished by using the polymerase chain reaction (PCR) (2), self-sustained sequence replication (3), or ligase amplification reaction (4, 5). Subsequently, single-base mismatches may be detected via allele-specific and reverse oligonucleotide hybridization (6, 7), denaturing gradient gel electrophoresis (8), RNase or chemical cleavage of mismatched heteroduplexes (9, 10), use of nucleotide analogs (11), or fluorescence PCR amplification/detection (12).

Landegren *et al.* (13) have pioneered an oligonucleotide ligation assay to circumvent the need for electrophoresis or precise hybridization conditions. Two oligonucleotide probes are hybridized to denatured DNA, such that the 3′ end of the first one is immediately adjacent to the 5′ end of the second probe. DNA ligase can covalently link these two oligonucleotides, provided that the nucleotides at the junction are perfectly base-paired to the target (4, 5, 13, 14). A single-nucleotide substitution can, therefore, be distinguished. Use of biotin on the first probe and a suitable nonisotopic reporter group on the second probe allows for product capture and detection (13) in a manner amenable to automation.

Ideally, the oligonucleotides should be sufficiently long (20–25 nucleotides) so that each will preferentially hybridize to its unique position on the human genome. The specificity of ligation should be particularly enhanced by performing the reaction at or near the melting temperature (t_m) of the two oligonucleotides. At higher temperatures a single-base mismatch at the junction forms not only an imperfect double helix but also destabilizes hybridization of the mismatched oligonucleotide.

This report describes DNA detection that uses a thermostable ligase to exquisitely discriminate between a mismatched and complementary DNA helix (Fig. 1 *Upper*). Because the enzyme retains activity after multiple thermal cycles, the ligations may be repeated to linearly increase product [termed ligase detection reaction (LDR)]. Product may be further amplified in a ligase chain reaction (LCR) by using both strands of genomic DNA as targets for oligonucleotide hybridization. Two sets of adjacent oligonucleotides, complementary to each target strand, are used. The ligation products from one round can become the targets for the next round of ligation (Fig. 1 *Upper*). By use of LCR, the amount of product can be increased in an exponential fashion by repeated thermal cycling.

MATERIALS AND METHODS

Thermostable Ligase. Plasmid libraries of *Thermus aquaticus* strain HB8 DNA (ATCC27634) were screened for the ability to complement a temperature-sensitive *lig*ts7 derivative of *Escherichia coli* [unpublished work; ref. 16]. One complementing plasmid (pDZ1) contained a thermostable ligase gene as evidenced by (*i*) presence of a thermostable NAD^+-dependent nick-closing (ligase) activity in crude extracts when assayed at 65°C (17) and (*ii*) DNA sequence analysis of the first 60 codons of the putative gene revealed >50% amino acid identity to *E. coli* ligase (18). Thermostable ligase was purified from *E. coli* cells containing the ligase gene cloned downstream of an inducible T7 expression system (19), as described elsewhere (unpublished work). Ligase activity was assayed for the ability to seal nicked plasmid DNA (pUC4KIXX) as monitored by electrophoresis on 1% agarose gel. One nick-closing unit of ligase is defined as the amount of ligase that circularizes 0.5 μg of nicked pUC4KIXX DNA in 20 μl of 20 mM Tris·HCl, pH 7.6/50 mM KCl/10 mM $MgCl_2$/1 mM EDTA/10 mM NAD^+/10 mM dithiothreitol overlaid with a drop of mineral oil after 15-min incubation at 65°C.

Genomic DNA, Plasmid DNA, and Oligonucleotides. Human genomic DNA was isolated from 0.5 ml of whole blood as described (20). Proteinase K and RNase A were removed by sequential extractions with phenol, phenol/chloroform, chloroform, 1-butanol (twice), and nucleic acid was recovered by precipitation with ethanol. Samples were boiled for 5 min before use in LCR assays. Plasmid DNAs containing the β^A- and β^S-globin gene alleles were a gift from D. Nickerson (California Institute of Technology, Pasadena, CA) and were digested with *Taq* I before use as target DNA. Oligonucleotides were assembled by the phosphoramidite method (21) on an Applied Biosystems model 380A DNA synthesizer, purified by reversed-phase HPLC, and provided

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Abbreviations: PCR, polymerase chain reaction; LDR, ligase detection reaction; LCR, ligase chain reaction.

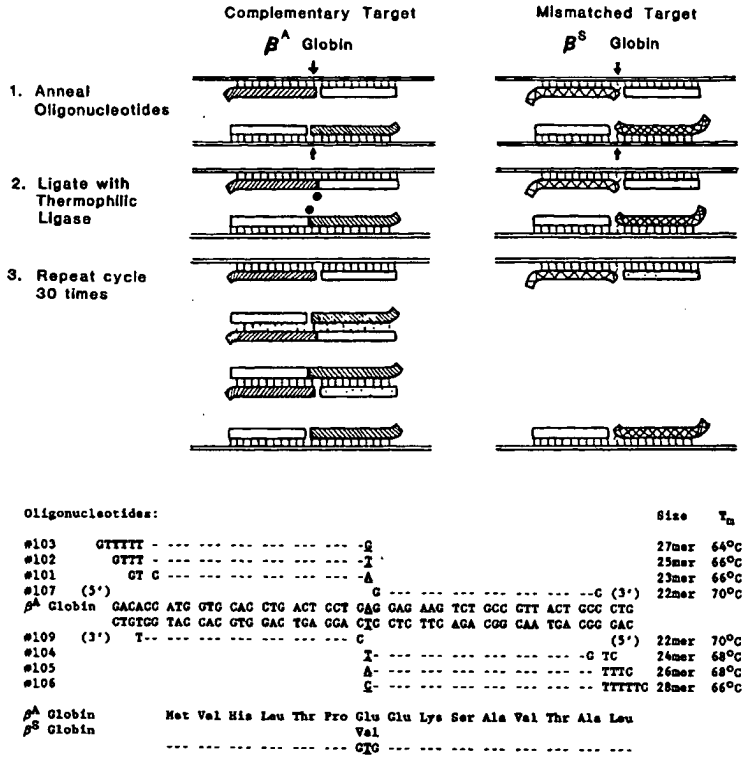

FIG. 1. (*Upper*) Diagram depicting DNA amplification/detection by using LCR. DNA is heat denatured, and four complementary oligonucleotides are hybridized to the target at a temperature near their melting temperature (65°C; t_m). Thermostable ligase will covalently attach only adjacent oligonucleotides that are perfectly complementary to the target (*Left*). Products from one round of ligations become targets for the next round, and thus products increase exponentially. Oligonucleotides containing a single-base mismatch at the junction do not ligate efficiently and, therefore, do not amplify product (*Right*). (*Lower*) Nucleotide sequence and corresponding translated sequence of the oligonucleotides used in detecting β^A- and β^S-globin genes. Oligonucleotides 101 and 104 detect the β^A target, whereas oligonucleotides 102 and 105 detect the β^S target when ligated to labeled oligonucleotides 107 and 109, respectively. Oligonucleotides 103 and 106 were designed to assay the efficiency of ligation of G–T or G–A and C–A or C–T mismatches when using β^A- or β^S-globin gene targets, respectively. Oligonucleotides have calculated t_m values of 66–70°C (15), just at or slightly above ligation temperature. The diagnostic oligonucleotides (101–106) contained slightly different length tails to facilitate discrimination of various products when separated on polyacrylamide denaturing gel.

by R. Kaiser and S. Horvath (California Institute of Technology, Pasadena, CA). Oligonucleotide sequences (5′–3′) are: 101, GTCATGGTGCACCTGACTCCTGA; 102, GTTTCATGGTGCACCTGACTCCTGT; 103, GTTTTTCATGGTGCACCTGACTCCTGG; 104, CTGCAGTAACGGCAGACTTCTCCT; 105, CTTTGCAGTAACGGCAGACTTCTCCA; 106, CTTTTTGCAGTAACGGCAGACTTCTCCC; 107, GGAGAAGTCTGCCGTTACTGCC; 109, CAGGAGTCAGGTGCACCATGGT. (See Fig. 1.)

^{32}P Labeling of Oligonucleotides. Oligonucleotides 107 or 109 (0.1 μg = 15 pmol) were 5′ end-labeled in 20 μl of 30 mM Tris·HCl, pH 8.0/20 mM Tricine/10 mM $MgCl_2$/0.5 mM EDTA/5 mM dithiothreitol/400 μCi of [γ-^{32}P]ATP (6,000 Ci/mM = 60 pmol ATP, New England Nuclear; 1 Ci = 37 GBq) by addition of 15 units of T4 polynucleotide kinase (New England Biolabs). After incubation at 37°C for 45 min, unlabeled ATP was added to 1 mM, and incubation was continued an additional 2 min at 37°C. The reaction was terminated by adding 0.5 μl of 0.5 M EDTA, and the kinase was heat-inactivated (65°C for 10 min). Unincorporated ^{32}P label was removed by chromatography with Sephadex G-25 preequilibrated with Tris/EDTA buffer. Specific activity ranged from 7 to 10 × 10^8 cpm/μg of oligonucleotide.

LDR and LCR Reaction Conditions. For LDR reactions, labeled oligonucleotide (200,000 cpm = 0.28 ng = 40 fmol) and unlabeled diagnostic oligonucleotide (0.27 ng = 40 fmol) were incubated in the presence of target DNA (1 fmol = 6 × 10^8 molecules of *Taq* I-digested β^A- or β^S-globin plasmid) in 10 μl of 20 mM Tris·HCl, pH 7.6/100 mM KCl/10 mM $MgCl_2$/1 mM EDTA/10 mM NAD^+/10 mM dithiothreitol/4 μg of salmon sperm DNA/15 nick-closing units of *T. aquaticus* ligase and overlaid with a drop of mineral oil. Reactions were incubated at 94°C for 1 min followed by 65°C for 4 min, and this cycle was repeated 5 or 20 times. For LCR reactions, unlabeled diagnostic oligonucleotide pairs (101 and 104, 102 and 105, or 103 and 106; 40 fmol each) and adjacent pairs of labeled oligonucleotides (107 and 109, 40 fmol each) were incubated in the presence of ligase and target DNA (ranging from 100 amol to less than one molecule per tube) with 20 or 30 cycles as described above.

Electrophoresis. Samples (4 μl) were in 45% formamide and denatured by boiling for 3 min before loading (40,000 or 80,000 cpm/lane). Electrophoresis was in 10% polyacrylamide gel containing 7 M urea in a buffer of 100 mM Tris borate, pH 8.9/1 mM EDTA for 2 hr at 60-W constant power. After removing urea, gels were dried and autoradiographed overnight at −70°C on Kodak XAR-5 film with the aid of a Cronex intensifying screen (DuPont).

RESULTS

The gene encoding human β-globin was selected as a model system to test ligation amplification and detection. The normal β^A and sickle β^S genes differ by a single A → T transversion that leads to a change of a glutamic acid residue to a valine in the hemoglobin β chain [Fig. 1, *Lower* (22)]. Diagnostic oligonucleotides containing the 3′ nucleotide unique to each allele were synthesized with different-length 5′ tails (Fig. 1 *Lower*). Upon ligation to the invariant ^{32}P-labeled adjacent oligonucleotide, the individual products could be distinguished when separated on a polyacrylamide denaturing gel and detected by autoradiography.

Specificity of Thermostable Ligase. The specificity of ligating oligonucleotide pairs on a target DNA with perfect complementarity was directly compared with each possible mismatch (see Fig. 2 and Table 1). Results show that *T. aquaticus* ligase efficiently links correctly base-paired oligonucleotides and gives near zero ligation in the presence of a mismatch (Table 1). When only 1 fmol of target DNA was used under LDR conditions, the worst mismatches were 1.5–1% (G–T, T–T), whereas other mismatches were <0.4% (A–A, C–T, G–A, G–A) of the products formed with complementary oligonucleotide base pairs (A·T). This is substan-

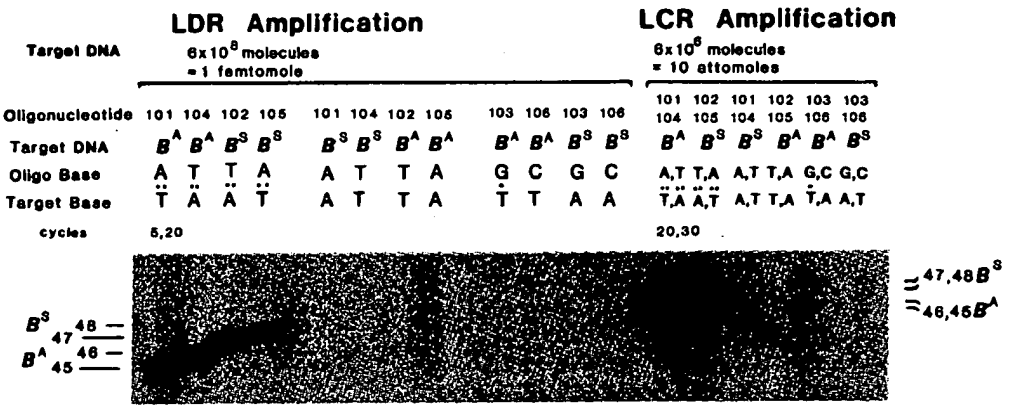

FIG. 2. Autoradiogram showing specificity of *T. aquaticus* ligase under LDR and LCR amplification conditions. Specificity was assayed by ligation of diagnostic oligonucleotides in the presence of either complementary or mismatched β^A- or β^S-globin gene target DNA (LDR amplification). Ligation of diagnostic oligonucleotides 101 (β^A allele), 102 (β^S allele), or 103 to labeled 107 gives lengths of 45, 47, or 49 nucleotides, respectively. For the complementary strand, ligation of diagnostic oligonucleotides 104 (β^A allele), 105 (β^S allele), or 106 to labeled 109 gives lengths of 46, 48, or 50 nucleotides, respectively. The diagnostic oligonucleotide listed in each lane and the appropriate adjacent labeled oligonucleotide (40 fmol each) was incubated with target DNA (1 fmol = 6×10^8 molecules of *Taq* I-digested β^A- or β^S-globin plasmid), as described. In LCR amplification, samples contained pairs of diagnostic oligonucleotides (β^A allele-specific 101 and 104, β^S allele-specific 102 and 105, or "C–G pair" 103 and 106), both labeled oligonucleotides (107 and 109), and were incubated with ligase and 10 amol of target DNA (6×10^6 molecules; 100-fold less than for LDR) as described. Samples were loaded in groups of eight and run into the gel; then the next set was loaded. This accounts for the "slower" migration of bands on the right side of the autoradiogram. (Intensifying screen was not used for this autoradiogram.) Bands were excised from the gel and assayed for radioactivity (Table 1).

tially better than found for mesophilic T4 or *E. coli* ligase when using similar radioactive detection methods (13, 14).

In the amplification/detection (LCR) experiments, four oligonucleotides were incubated with ligase and 10 amol of target DNA (see Fig. 2 *Right* and Table 1 lower part). The 3′ nucleotide of each unlabeled diagnostic oligonucleotide was either complementary or mismatched to the target DNA and yet was always complementary to its pair—i.e., A·T for 101 and 104, T·A for 102 and 105, and G·C for 103 and 106.

Table 1. Quantitation of complementary and mismatched LDR and LCR

Amplification	Oligonucleotide base–target base	Product formed, %*	Mismatched/ complementary, %†
LDR (6×10^8 target molecules = 1 fmol)	A–T	21.5	
	T–A	13.2	
	T–A	17.9	
	A–T	12.4	
	A–A	<0.1	<0.4
	T–T	0.12	0.7
	T–T	0.16	1.0
	A–A	<0.1	<0.4
	G–T	0.30	1.4
	C–T	<0.1	<0.4
	G–A	<0.1	<0.4
	C–A	<0.1	<0.4
LCR (6×10^6 target molecules = 10 amol)	A–T, T–A	41.4	
	T–A, A–T	10.4	
	A–A, T–T	0.45	1.1
	T–T, A–A	<0.05	<0.2
	G–T, C–A	0.51	1.3
	G–A, C–T	<0.05	<0.2

Bands from 20-cycle LDR and 30-cycle LCR experiments described in Fig. 2 were excised from the gels and assayed for radioactivity.

*Percentage product formed = cpm in product band/cpm in starting oligonucleotide band.

†Percentage mismatched/complementary = cpm in band of mismatched oligonucleotide/cpm in band of complementary oligonucleotide when using the same target DNA and indicates noise-to-signal ratio.

Four-way (target independent) ligation was minimized by use of (*i*) carrier salmon sperm DNA and (*ii*) oligonucleotides designed to create single-base 3′ overhangs (this work, see Fig. 1) or single-base 5′ overhangs (not tested). Note that an initial "incorrect" ligation of a mismatched oligonucleotide to target DNA would subsequently be amplified with the same efficiency as a correct ligation (See Fig. 1). Nevertheless, the worst mismatches were 1.3% to 0.6% (G–T, C–A; A–A, T–T), whereas others were <0.2% (T–T, A–A; G–A, C–T) of the products formed with complementary basepairs (A·T, T·A). LCR, using thermostable ligase, is thus the only method that can both amplify and detect single-base mismatches with high signal-to-noise ratios (4, 5).

The entire set of experiments described above was repeated with a buffer containing 150 mM instead of 100 mM KCl. Results were essentially the same as in Fig. 2 and Table 1; mismatches for LDR ranged from 0.6% to <0.3% and for LCR ranged from 1.7% to <0.3% of the complementary products (data not shown). Thus for *T. aquaticus* ligase, discrimination between matched and mismatched oligonucleotides is not critically dependent on salt conditions, in contrast to the requirements for mesophilic ligases (4, 5, 13, 14).

Specificity of LCR DNA Amplification with Sub-amol Quantities of Target DNA. The extent of LCR DNA amplification was determined in the presence of target DNA ranging from 100 amol = 6×10^7 molecules to <1 molecule per tube (Fig. 3, Table 2). In the absence of target DNA, no background signal was detected when carrier salmon sperm DNA (4 μg) was present (compare last 8 lanes of Fig. 3). At higher target concentration, DNA amplification was essentially complete after 20 cycles, whereas at lower initial target concentration substantially more product is formed with additional amplification cycles. After 30 cycles of LCR, 200 molecules of initial target DNA were amplified 1.7×10^5 fold and thus could be readily detected. The average efficiency of ligation per cycle (40–50%, calculated as described in ref. 4) could be potentially enhanced by altering buffer conditions [such as using NH_4Cl, $MnCl_2$, polyamines, or polyethylene glycols (17)], enzyme concentration, or thermal-cycling times and temperatures.

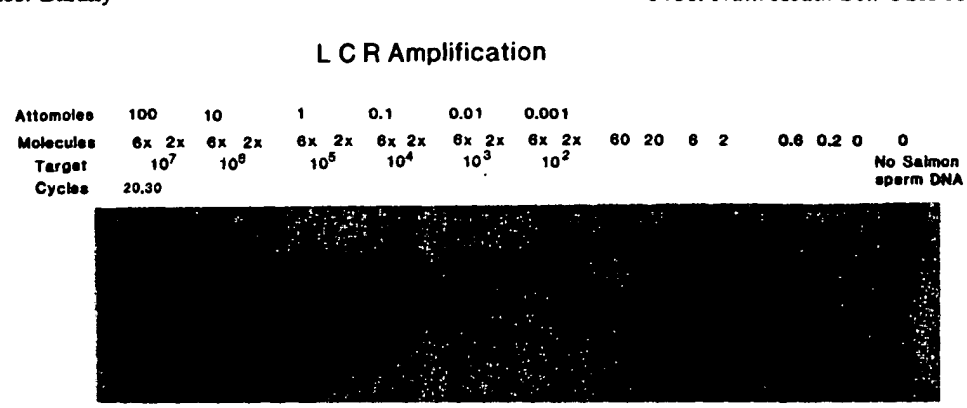

FIG. 3. Autoradiogram showing LCR amplification at different target concentrations. Labeled invariant oligonucleotides (107 and 109; 200,000 cpm = 40 fmol each) and unlabeled β^A allele oligonucleotides (101 and 104; 40 fmol each) were incubated with target DNA (ranging from 100 amol = 6×10^7 molecules to <1 molecule per tube of *Taq* I-digested β^A-globin plasmid) and ligase as described. Samples were electrophoresed, gel was autoradiographed overnight, and bands were counted as described (see Table 2). Bands of 45 and 46 nucleotides correspond to ligation products of the coding and complementary β^A-globin oligonucleotides. Lower-molecular-mass products correspond to ligation of minor species in the synthesized oligonucleotide preparations that were shorter than intended product. Samples were loaded in groups of eight, giving the appearance of slower migration on the right of the autoradiogram.

To test ligase discrimination between complementary and mismatched oligonucleotides in a direct competition assay, the above LCR experiment was repeated with or without oligonucleotides that would give G–T and C–A mismatches (see Table 3). At higher target concentrations, the mismatched product ranged from 1.8% to 0.5% of the complementary product. Mismatched product could not be detected when using <3 amol of target DNA. As control, excess mismatched target DNA (β^S- instead of β^A-globin DNA at 6×10^7 molecules per tube) gave only 2.1% and 1.5% product. Thus, the signal from the correctly paired ligation products is 50- to 500-fold higher than from mismatched products, under either competition or individual LCR ligation conditions.

Detection of β-Globin Alleles in Human Genomic DNA. DNA isolated from the blood of normal ($\beta^A\beta^A$), carrier ($\beta^A\beta^S$), and sickle cell ($\beta^S\beta^S$) individuals was tested for allele-specific LCR detection. With target DNA corresponding to 10 μl of blood, β^A and β^S alleles could be readily detected by using allele-specific LCR (Fig. 4). As seen with plasmid-derived target DNA (see Fig. 2), efficiency of ligation (and hence detection) is somewhat less for β^S- than β^A-specific oligonucleotides. This difference may be a function of the exact nucleotide sequence at the ligation junction or the particular oligonucleotides (with differing 5′ tails) used in these LCR experiments. Nevertheless, the results show the feasibility of direct LCR allelic detection from blood samples without any need for primary PCR or self-sustained sequence replication amplification.

Table 2. Quantitation of LCR amplification

Target molecules	Product formed, %*	Amplification†
6×10^7	134‡	
2×10^7	96	
6×10^6	107‡	
2×10^6	78	
6×10^5	85	
2×10^5	48	5.8×10^4
6×10^4	25	1.0×10^5
2×10^4	4.5	5.4×10^4
6×10^3	2.3	9.2×10^4
2×10^3	0.36	4.3×10^4
6×10^2	0.18	7.2×10^4
2×10^2	0.14	1.7×10^5
$60 \rightarrow 0$§	<0.05	

Bands from 30-cycle LCR experiment described in Fig. 3 were excised from gels and assayed for radioactivity.
*Percentage product formed = cpm in product band/cpm in starting oligonucleotide band.
†Amplification = no. of product molecules formed/no. of target molecules.
‡At higher target concentration, DNA amplification was essentially complete after 20 cycles; slightly imprecise excision of 30-cycle bands from this portion of the gel probably accounts for product formed values >100%.
§Product formed from 0 to 60 target molecules was indistinguishable from background (see Fig. 3).

DISCUSSION

The specificity, yield, and sensitivity of PCR were significantly improved by incorporating use of a thermostable DNA polymerase (2), resulting in a simplified procedure that has

Table 3. Quantitation of LCR amplification with or without mismatched competitor oligonucleotide

	Complementary oligonucleotides	Complementary and mismatched oligonucleotides	
Target molecules	Product formed, %*	Product formed, %*	Mismatched/ complementary, %†
6×10^7 (β^A)	114*	93	1.0
2×10^7 (β^A)	93	95	1.8
6×10^6 (β^A)	102*	93	0.5
2×10^6 (β^A)	90	67	0.5
6×10^5 (β^A)	51	46	
2×10^5 (β^A)	31	23	
6×10^4 (β^A)	17	9.3	
2×10^4 (β^A)	8.6	2.9	
6×10^3 (β^A)	3.2	0.8	
0	<0.1	<0.1	
6×10^7 (β^S)	2.1	1.5	

One set of experiments contained 40 fmol each of β^A allele oligonucleotides 101 and 104 per tube, exactly as described for Fig. 3, whereas the second set had, in addition, 40 fmol each of oligonucleotides 103 and 106 per tube (forming G–T and C–A mismatches, respectively). Bands from 30-cycle LCR experiment, as described for Fig. 3, were excised from the gels and assayed for radioactivity.
*Percentage product formed = cpm in complementary product band/cpm in starting oligonucleotide band. Imprecise excision of two bands from the gel probably accounts for product formed values >100% (see Table 2).
†Percentage mismatched/complementary = cpm in bands of mismatched oligonucleotide products/cpm in band of complementary oligonucleotide products in same lane and indicates noise-to-signal ratio.

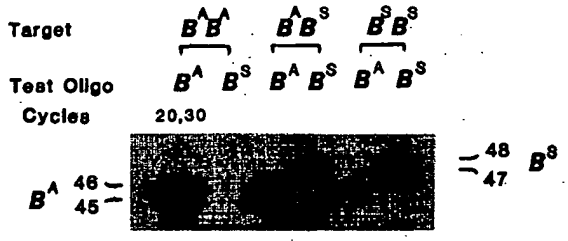

FIG. 4. Detection of β-globin alleles in human genomic DNA by autoradiogram. DNA was isolated from blood samples of normal ($\beta^A\beta^A$), carrier ($\beta^A\beta^S$), and sickle cell ($\beta^S\beta^S$) individuals as described. Genomic DNA (corresponding to 10 μl of blood or ≈ 6×10^4 nucleated cells) was tested in two separate tubes containing labeled oligonucleotides (107 and 109; 200,000 cpm = 40 fmol each) and either unlabeled β^A test oligonucleotides (101 and 104) or unlabeled β^S test oligonucleotides (102 and 105; 40 fmol each). Both reaction mixtures were incubated under the same buffer (without salmon sperm DNA), enzyme, and cycle conditions described. Samples were electrophoresed, and the gel was autoradiographed overnight as described. Ligation products of 45 and 46 or 47 and 48 nucleotides indicate presence of the β^A- or β^S-globin gene, respectively. Oligo, oligonucleotide.

become widely applicable (23, 24). Similarly, this report demonstrates the utility of thermostable ligase for allelic-specific gene detection under both LDR and LCR conditions. Both LCR and PCR amplification derive their specificity from the initial hybridization of primer to target DNA, and this is enhanced by (*i*) use of oligonucleotides of sufficient length to be unique in the human genome and (*ii*) use of temperatures near the oligonucleotide t_m. LCR amplification faithfully detected as few as 200 initial target molecules, as well as both β^A and β^S alleles directly from genomic DNA. LCR did not amplify a T–T, G–T, C–T, or C–A 3′-terminal mismatch, as has been reported for allele-specific PCR amplifications (25). Whether LCR will tolerate internal mismatches present in viral variants remains to be determined (25).

LCR amplification/detection is compatible with a primary amplification of genomic DNA by either PCR (2) or self-sustained sequence replication (3). Such a primary amplification could allow for LCR detection of emerging viral subpopulations where the mutations are known, such as the multiple mutations in human immunodeficiency virus conferring resistance to 3′-azido-3′-deoxythymidine (AZT) (26). One can also envisage multiplexing the primary amplification of dozens of loci simultaneously (27) and aliquoting products into separate microtiter wells. A subsequent round of LCR amplification/detection could then distinguish a particular target loci, even if it were initially amplified only in the amol range. Such a multiplex PCR/LCR detection assay, with the potential for an automated format, could (*i*) rapidly screen large populations for monogenic disease polymorphisms, (*ii*) distinguish several polymorphisms simultaneously from a single sperm to map the relative positions of these polymorphisms (28), and (*iii*) help eliminate current ambiguities in DNA identification of individuals for forensic or paternity cases (29).

The potential uses of thermostable enzymes that survive the temperature-cycling conditions required to denature double-stranded DNA are just now being tapped. With variations of the LCR concepts outlined above, thermostable ligase could be used to (*i*) covalently capture specific DNA fragments to a solid matrix, with the aid of "template oligonucleotides" (40- to 50-mers) complementary to both the fragment end as well as a second oligonucleotide attached to a solid support, (*ii*) covalently link PCR-generated fragments (for example, protein domains or exons) in specific order, and (*iii*) covalently link two members of a hexamer oligonucleotide library to form specific dodecamers for directed sequencing of cosmids and other large DNAs (30).

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Thermophilic DNA Ligase

PURIFICATION AND PROPERTIES OF THE ENZYME FROM *THERMUS THERMOPHILUS* HB8*

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Thermophilic and thermostable DNA ligase was purified to near homogeneity from the extract of *Thermus thermophilus* HB8. The purified enzyme has an isoelectric point at pH 6.6 and consists of a single polypeptide of about 79,000 in molecular weight on the bases of sodium dodecyl sulfate-polyacrylamide gel electrophoresis data and an equilibrium sedimentation method. The enzyme requires divalent cations, Mg^{2+} or Mn^{2+}, and the optimum concentration of these ions being 5–9 × 10^{-3} M and 3–6 × 10^{-3} M, respectively. The enzyme also requires NAD as a cofactor. The apparent K_m for NAD is 1.85 × 10^{-5} M and that of $(dT)_{10}$ is 1.4 × 10^{-4} M. The pH optimum is 7.4–7.6 in Tris-HCl and 8.0 in collidine/HCl buffer. The joining reaction is activated by K^+ and NH_4^+ at a concentration of 2–100 mM and inhibited by Na^+ above 25 mM. The optimum temperatures of the joining of thymidylate oligomers in the presence of poly(dA) as a template are 27.5 °C for $p(dT)_8$, 34.5 °C for $p(dT)_{10}$, and 37 °C for $p(dT)_{12-18}$ and that of cohesive-end DNA restriction fragments is 24–37 °C. The nick-closing activity of the enzyme was observed over a wide range of the temperature from 15 to 85 °C and the optimum temperature is 65–72 °C. The temperature dependency of ligation with HB8 DNA ligase for various substrates was found to shift to a region of 7–10 °C higher than that of T4 DNA ligase and the activity of HB8 DNA ligase decreased remarkably below 4 °C. The enzyme was stable for 1 week at 37 °C, its activity dropped by 50% within 2 days at 65 °C.

DNA ligases catalyze the formation of phosphodiester linkages between DNA chains. They have been isolated from a variety of sources. In particular the enzymes from *Escherichia coli* and T4 phage have been studied extensively (1–4).

Recently, various enzymes from thermophilic bacteria have been isolated such as DNA-dependent RNA polymerase (5) and nuclease TT1 (6) from *Thermus thermophilus* HB8 and restriction endonucleases from *T. thermophilus* 111 (7, 8). These enzymes are thermostable and have high optimum temperatures as expected in consideration of their source. An attempt was thus made to find a new thermostable and thermophilic DNA ligase in thermophilic bacteria. It was worth-while to determine whether the ligation of a thermophilic DNA ligase would proceed at a high temperature, considering that individual melting temperatures of DNA fragments restricted to the optimum temperatures of ligation.

This paper describes the purification and properties of a DNA ligase from *T. thermophilus* HB8, which was isolated in a nearly homogeneous form and termed HB8 DNA ligase. The optimum temperatures for various substrates are compared with those of T4 DNA ligase.

EXPERIMENTAL PROCEDURES[1]

RESULTS

Purity and Molecular Weight

Samples at each step of purification in Table I were subjected to SDS[2]-polyacrylamide gel electrophoresis. A single protein band was observed in steps 7 and 8 after staining with Coomassie Brilliant Blue. Although several minor bands were detected on the same gel after successive silver staining, the purity of the enzyme was considered more than 95% (Fig. 1). Following disc gel electrophoresis of the purified enzyme in 7.5% nondenatured polyacrylamide gels, DNA ligase activity was extracted from 1-mm slices of one of these gels. The position of the activity coincided with that of a single protein band stained with Coomassie Brilliant Blue (Fig. 2).

To check for contamination from nucleases, $[5'\text{-}^{32}P](dT)_{10}$ without poly(dA) was incubated with the purified enzyme and subjected to polyacrylamide gel electrophoresis. Hydrolysis of the substrate in the presence of 100 times of the amount of the enzyme used for general assay could not be detected.

The molecular weight of HB8 DNA ligase was determined on the basis of its mobility in 10 or 12.5% of SDS-polyacrylamide gel electrophoresis (15) as 78,500: using the sedimentation equilibrium method (18), the natural logarithm of the concentration (C) of HB8 DNA ligase was plotted as a function of the square of the distance (γ^2) from the center rotation (Fig. 3). When the partial specific volume was assumed to be 0.73, a molecular weight of this enzyme was calculated to be 80,400 from the slope of $d\ln C/d\gamma^2$ in Fig. 3. From these results HB8 DNA ligase was considered to have a molecular weight about 79,000 and to consist of a single polypeptide.

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[1] Portions of this paper (including "Experimental Procedures" and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M565, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviation used is: SDS, sodium dodecyl sulfate.

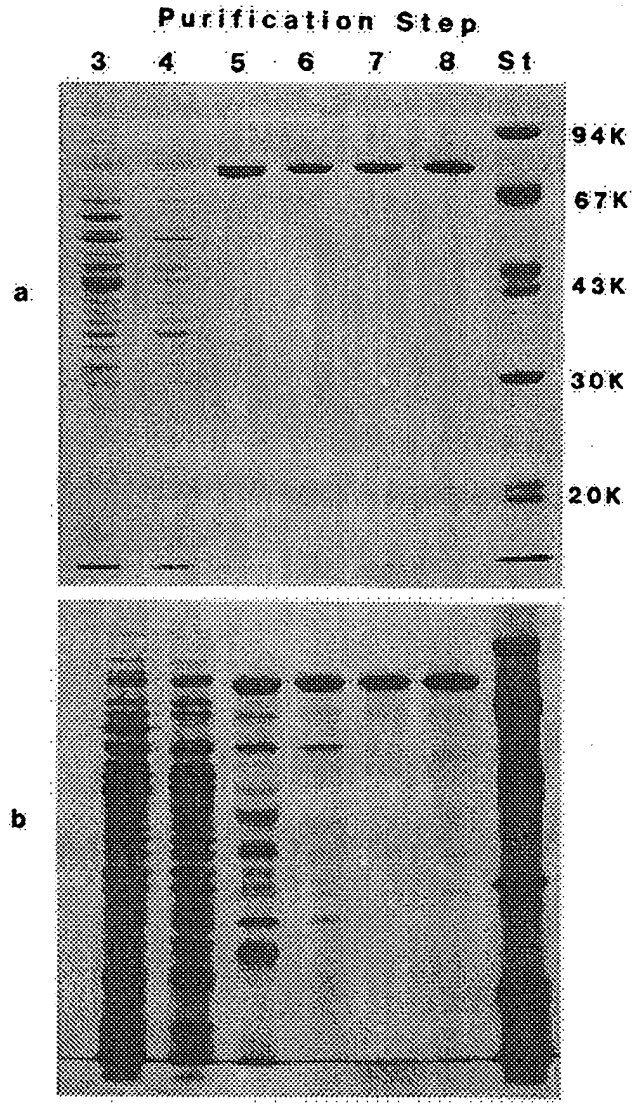

FIG. 1. **SDS-polyacrylamide gel electrophoresis of HB8 DNA ligase.** The 10% SDS-polyacrylamide gel electrophoresis was performed with the enzyme of each purification step in Table I. The amount of protein ($\times 10^{-3}$ A_{260} units) applied to each slot is as follows: *slot 3*, 15; *slot 4*, 16; *slot 5*, 4.5; *slot 6*, 3.2; *slot 7*, 3.7; *slot 8*, 2.0. As standard proteins (about 5 μg each) a low molecular weight calibration kit (Pharmacia Fine Chemicals) was used. Numerals indicate molecular weights in thousands. *a*, Coomassie Brilliant Blue staining; *b*, silver staining.

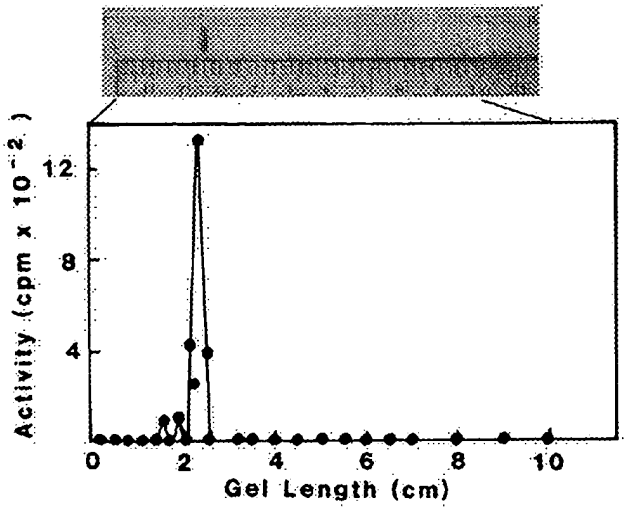

FIG. 2. **Polyacrylamide disc gel electrophoresis and HB8 DNA ligase activity.** The purified HB8 DNA ligase (2.0×10^{-3} A_{260} units) was electrophoresed in 7.5% polyacrylamide gels and the position of DNA ligase activity was determined. Detailed conditions are described under "Methods".

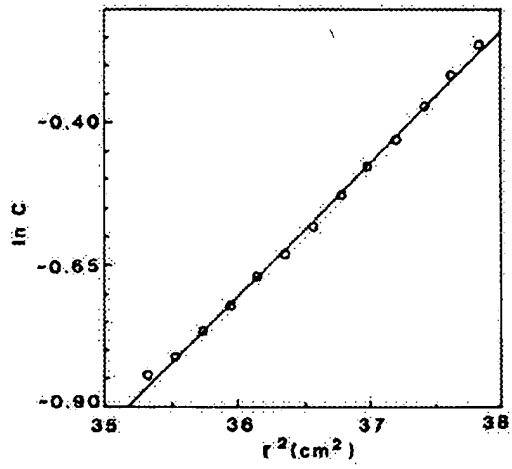

FIG. 3. **Equilibrium sedimentation of HB8 DNA ligase.** The purified enzyme (0.413 A_{260}/ml) in 20 mM Tris-HCl, pH 7.6, containing 0.1 M KCl and 0.01 M $MgCl_2$ was equilibrated at rotor speeds of 6942 rpm in a Hitachi 282 analytical ultracentrifuge using a RA-72TC rotor. The natural log of the enzyme concentration (C) was plotted as a function of the square of the distance (r^2) from the center of rotation.

Catalytic Properties

As shown in Table II, HB8 DNA ligase required NAD as a cofactor, which could not be substituted for by ATP. Although some enzyme activity was observed without NAD, this is due to the fact that some of the enzyme-AMP complex remained in the original extract. The enzyme required Mg^{2+} as a divalent cation, but did not require a sulfhydryl reagent such as dithiothreitol. When the substrate was oligo(dT), poly(dA) was necessary as a complementary strand.

The ligation of HB8 DNA ligase had an optimum pH range of 7.4–7.6 in Tris-HCl and an optimum pH of 8.0 in collidine/HCl buffer. The optimum concentration of Mg^{2+} was 5–9 $\times$ 10^{-3} M and activity increased by about twice as much when Mn^{2+} was substituted for Mg^{2+} at levels of 3–6 $\times$ 10^{-3} M (Fig. 4*a*). A low concentration of monovalent cations (1–150 mM), K^+, and NH_4^+, markedly stimulated the joining reaction of HB8 DNA ligase, but a low level (20–150 mM) of Na^+ inhibited the enzyme activity (Fig. 4*b*).

TABLE II

Requirements of HB8 DNA ligase

Enzyme activity was assayed as described under "Methods"

Components	Activity	Relative activity
	pmol	
Complete	60.7	100
−NAD	4.0	6.6
−NAD, +ATP[a]	2.7	4.4
−$MgCl_2$	0	0
−Dithiothreitol	68.2	112
−Poly(dA)	0	0

[a] NAD was replaced with ATP at the same concentration.

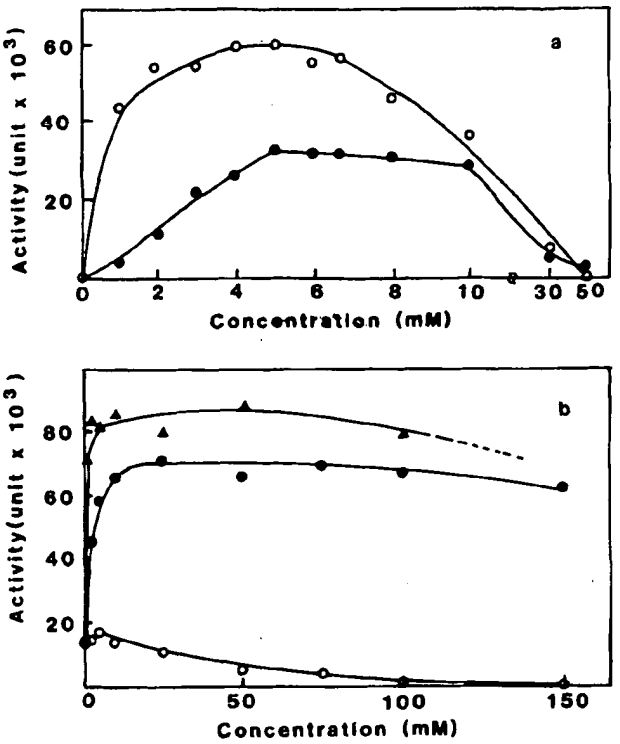

FIG. 4. **Effects of cations on HB8 DNA ligase activity.** *a*, effects of Mg^{2+} and Mn^{2+}. The enzyme was assayed at various concentrations of $MgCl_2$ (●) or the substitution of $MnCl_2$ for $MgCl_2$ (○) under the conditions described under "Methods". *b*, effects of monovalent cations. The enzyme was assayed at various concentrations of KCl (●), NaCl (○), or NH_4Cl (▲) under the conditions described under "Methods".

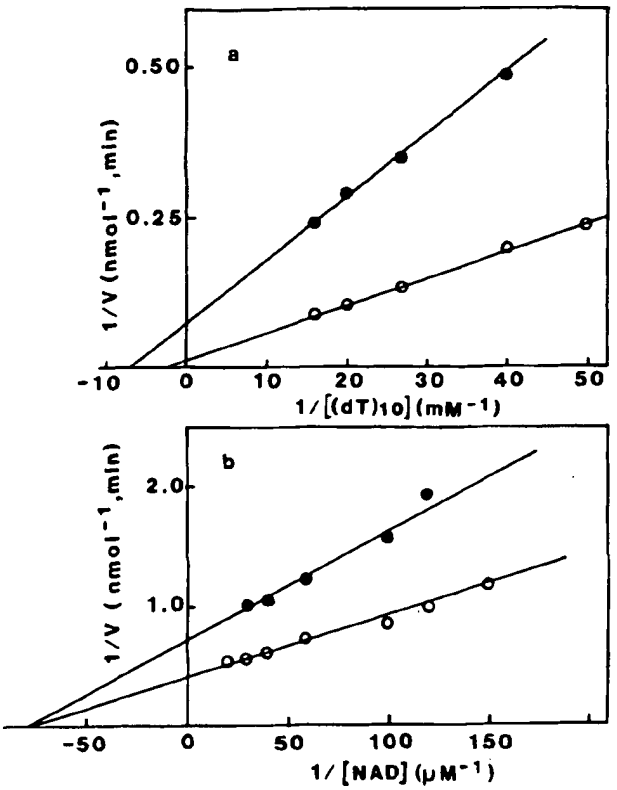

FIG. 5. **Kinetics of HB8 DNA ligase and effect of KCl.** *a*, double-reciprocal plots of initial velocities *versus* substrate concentrations. Concentration of $p(dT)_{10}$ was varied (●), plus 50 mM KCl, (○). *b*, double-reciprocal plots of initial velocities *versus* NAD concentrations. Concentration of NAD was varied (●), plus 50 mM KCl (○).

In Fig. 5*a*, double reciprocal plots of initial velocities *versus* concentrations of $p(dT)_{10}$ are shown along with the effects of KCl on the kinetic parameters. K_m for the joining of $p(dT)_{10}$ on poly(dA) was found to be 1.4×10^{-4} M. The presence of K^+ (50 mM) increased the V_{max} by about 5 times. K_m for NAD was 1.85×10^{-3} M and not affected by the presence of K^+ (Fig. 5*b*). These results suggest that K^+ has no effect on the rate of formation of the ligase-AMP complex and stimulates the rate of dissociation of the enzyme-adenylate from $p(dT)_{10}$ as reported by Modrich and Lehman (20). The purified enzyme retained its complete activity at 24 and 37 °C for 1 week and 50% of its activity at 65 °C for 2 days (Fig. 6). The enzyme was stable at 4 °C for several months and could be stored at −80 °C.

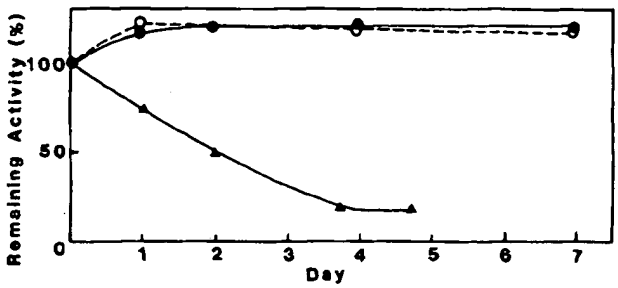

FIG. 6. **Temperature-stability of HB8 DNA ligase.** The enzyme solutions were kept at 24 °C (●), 37 °C (○), and 65 °C (▲) for several days. At arbitrary intervals, the remaining activity was measured.

Temperature Dependency of Ligation with HB8 DNA Ligase

Joining of Various Oligonucleotides—Fig. 7 shows the relative joining rates of thymidylate oligomers in the presence of poly(dA) at various temperatures with both HB8 and T4 DNA ligases. As in the case of T4 DNA ligase (21), the optimum temperature of HB8 DNA ligase varies with the length of the strand. The enzyme joined $p(dT)_8$ most readily at 27.5 °C, $p(dT)_{10}$ at 34 °C, and $p(dT)_{12-18}$ at 37.5 °C. This confirms that the temperature optimum of HB8 DNA ligase is 7–10 °C higher than that of T4 DNA ligase regardless of substrate length. Fig. 8 shows the time course of ligation of $p(dT)_{10}$ at different temperatures (*a*) and ligation products at various temperatures (*b*) with HB8 DNA ligase. The optimum temperature did not change as the reaction proceeded. The ligation products at lower temperatures (22 and 30 °C) were found to be shorter than those at higher temperatures (40 and 45 °C).

A comparison of the joining rates of various oligomers at 37 °C is given in Table III. The joining rate decreased greatly when a thymidylate oligomer had chain length below 8. Among the oligomers with chain lengths of 12–18, oligo(dA) on poly(dT) and oligo(dG) on poly(dC) were joined at only a fraction of the rate that oligo(dT) was joined on poly(dA) as a complementary strand. Substitution of poly(rA) for poly(dA) as a template for $p(dT)_{10}$ decreased the joining rate to the point where it was negligible.

Ligation of Nicked DNA—Time course and temperature dependency of the nick-closing activity of HB8 DNA ligase were investigated on form II of PM2 DNA (Fig. 9). The formation of form I DNA was observed over a wide range of temperatures from 15 to 85 °C and the optimum temperature

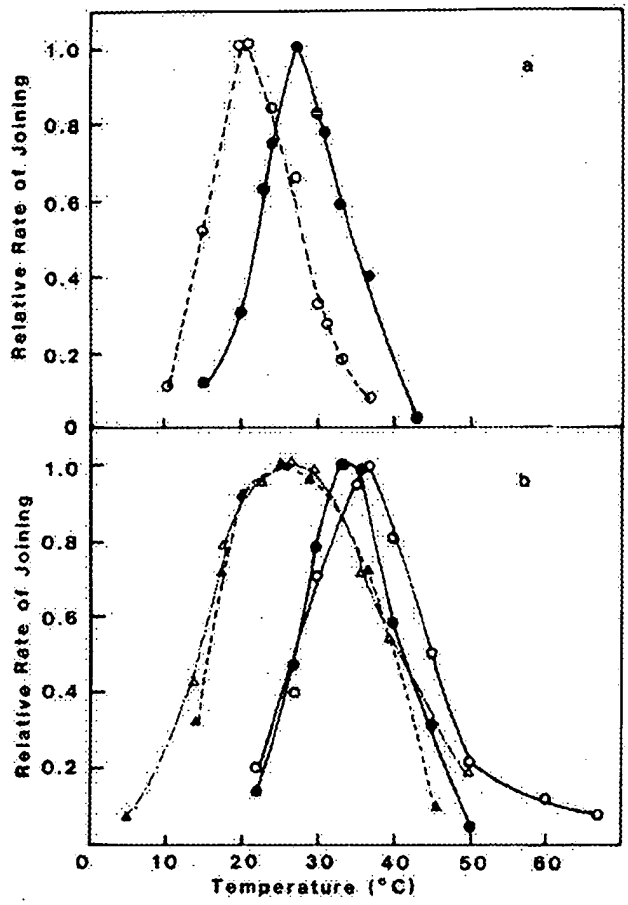

FIG. 7. **Relative joining rate of various oligo(dT) on poly(dA) at different temperatures.** *a*, $p(dT)_8$ on poly(dA): HB8 DNA ligase (●), T4 DNA ligase (○); *b*, $p(dT)_{10}$ on poly(dA): HB8 DNA ligase (●), T4 DNA ligase (▲); $p(dT)_{12-18}$ on poly(dA): HB8 DNA ligase (○), T4 DNA ligase (△), assay conditions are described under "Methods". In the case of T4 DNA ligase, NAD was replaced with ATP at the same concentration.

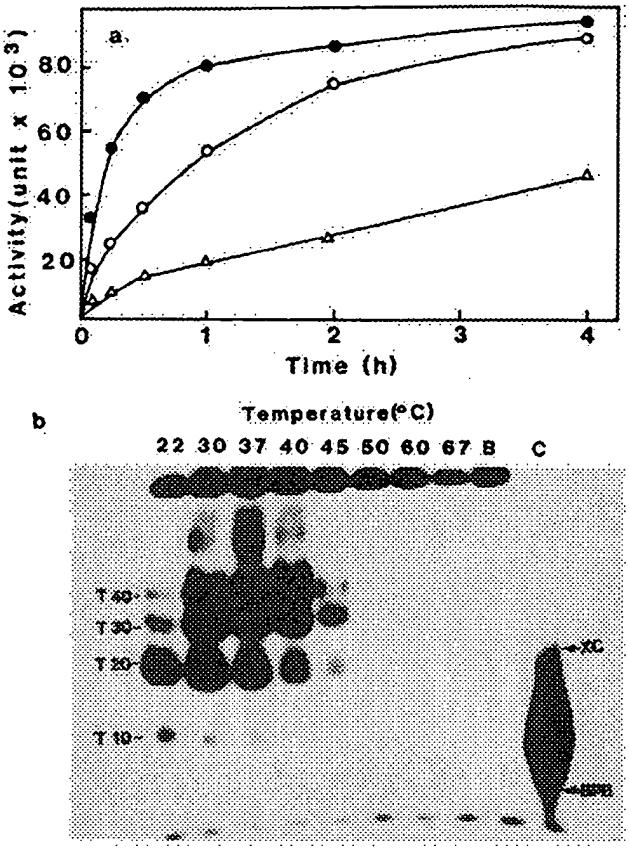

FIG. 8. **Ligation of ^{32}P-$(dT)_{10}$ on poly(dA) at various temperatures by HB8 DNA ligase.** *a*, time course of ligation at 26.5 (○), 36 (●), and 43 °C (△). *b*, autoradiogram of reaction products at various temperatures on a 20% polyacrylamide gel containing 8 M urea. *Slot B* shows the reaction product without the enzyme and *slot C* shows the position of ^{32}P-$(dT)_{10}$. Detailed conditions are described under "Methods".

was 65–72 °C. Some of the results are shown in Fig. 9*b*.

Ligation of Restriction Endonuclease-cleaved DNA—Ligation of *Hin*dIII-cleaved λDNA fragments with HB8 DNA ligase was examined at various temperatures. The ligation activity was highest at 24–37 °C. The result are shown in part in Fig. 10*a*. At optimum temperature, HB8 DNA ligase was examined for its ability to join both cohesive and blunt-end DNA fragments. As shown in Fig. 10*b*, cohesive-end DNA fragments with various restriction enzymes were easily ligated. The recutting with the restriction endonucleases converted the respective ligation products back to the initial length of DNA fragments. The fact proved that *Hin*dIII, *Pst*I, *Eco*RI, and *Mlu*I sites are actually being joined in the cohesive-end ligation, respectively. However, in case of the ligation of blunt-end DNA fragments such as *Hpa*I- and *Sma*I-digested λDNA, HB8 DNA ligase was unable to join any one of them (data not shown).

DISCUSSION

DNA ligase was purified almost homogeneously from the cell extract of an extreme thermophile, *T. thermophilus* HB8. The molecular weight of the enzyme was determined to be about 79,000. Most procaryotic DNA ligases require NAD as a cofactor and thus, HB8 DNA ligase is highly specific for NAD. A part of the crude enzyme showed the activity in the absence of NAD and after NMN treatment the enzyme required the cofactor. Also, NMN treatment of the enzyme in a purification step improved the affinity for DNA-cellulose column. These results indicate that some of the enzyme takes on adenylated forms in the crude extract. The enzyme requires a divalent cation, Mg^{2+} or Mn^{2+} for activity, and the enzymatic activity was markedly stimulated with low concentrations of monovalent cations, NH_4^+ and K^+, not Na^+. These properties are very similar to *E. coli* DNA ligase. The catalytic properties of the ligation of deoxyribooligonucleotides with the complementary polymers in Table III are also similar to *E. coli* DNA ligase rather than T4 DNA ligase. HB8 DNA ligase has a large K_m for the joining of $p(dT)_{10}$ on poly(dA) compared with the reported K_m values for similar substrates toward *E. coli* and T4 DNA ligases (4).

TABLE III

HB8 DNA ligase activity on deoxyribooligomers

Enzyme activity was assayed as described under "Methods"

Substrate	Template	Activity at 37 °C	
		nmol/ml enzyme	%
$p(dT)_8$	poly(dA)	5.6	1.8
$p(dT)_{10}$	poly(dA)	300	100
$p(dT)_{10}$	poly(rA)	NG*	NG
$p(dT)_{12-18}$	poly(dA)	279	90
$p(dA)_{12-18}$	poly(dT)	0.6	0.2
$p(dG)_{12-18}$	poly(dC)	1.2	0.4

*NG, the ligation is negligible.

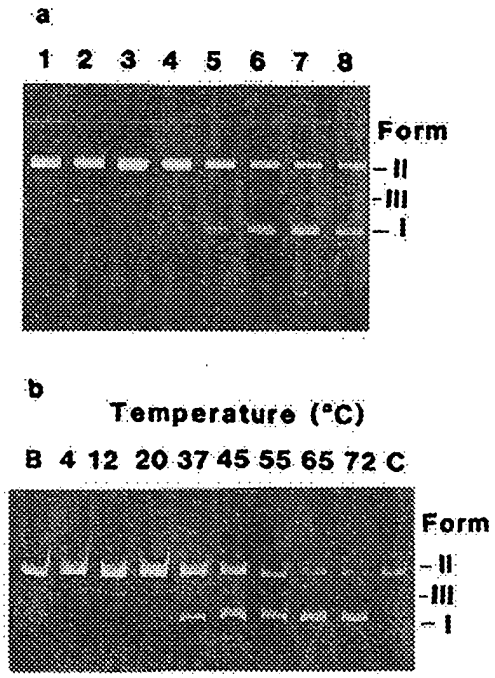

FIG. 9. **Agarose gel electrophoresis of PM2 form II DNA incubated with HB8 DNA ligase.** *a*, time course of nick-closing activity: PM2 form II DNA was incubated with HB8 DNA ligase (1.73×10^{-4} units) at 65 °C for 2 min (*slot 2*), 5 min (*slot 3*), 10 min (*slot 4*), 30 min (*slot 5*), 1 h (*slot 6*), 2 h (*slot 7*), and 4 h (*slot 8*). *Slot 1* was incubated without the enzyme for 5 min. Electrophoresis in a horizontal 0.7% agarose gel (6 × 6 × 0.6 cm) was carried out at 50 V for 2 h. *b*, temperature dependency of nick-closing activity: PM2 from II DNA (about 0.5 μg) was incubated with HB8 DNA ligase (1.73×10^{-5} units) at various temperatures for 15 min. *Slot B* was incubated without the enzyme. *Slot C* is standard of PM2 DNA form I, II, and III in a 0.7% agarose gel. Detailed conditions are described under "Methods".

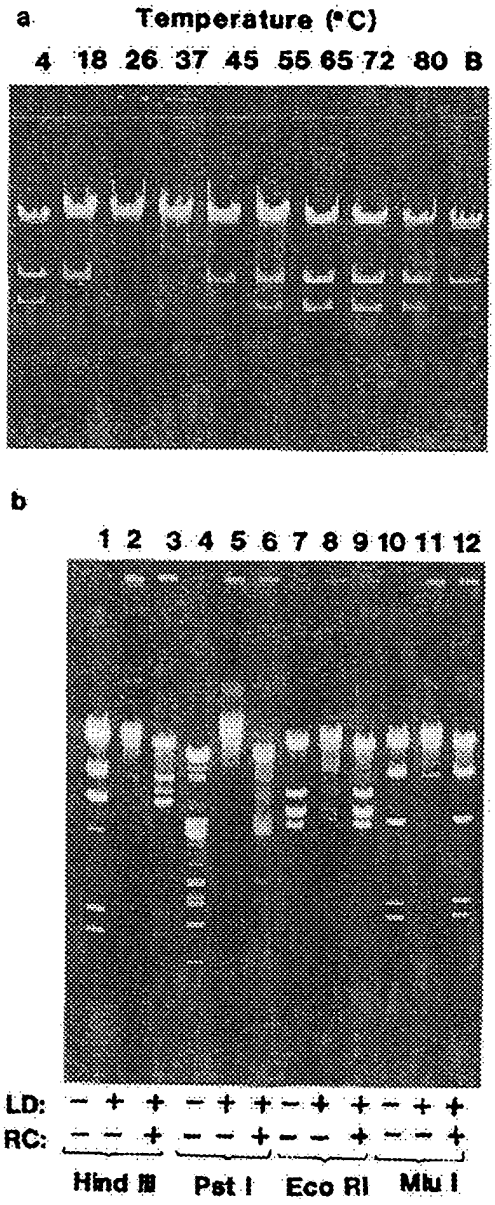

FIG. 10. **Agarose gel electrophoresis of ligation products by HB8 DNA ligase.** *a*, temperature dependency of cohesive-end ligation: *Hin*dIII-digested λDNA was incubated with HB8 DNA ligase (3.46 units) at various temperatures for 2 h. The products were analyzed by 0.9% agarose gel electrophoresis. *Slot B* shows incubation without the enzyme at 37 °C. *b*, ligation of various cohesive ends and recutting by the same restriction enzymes: *Hin*dIII (*slots 1, 2,* and *3*)-, *Pst*I (*slots 4, 5,* and *6*)-, *Eco*RI (*slots 7, 8,* and *9*)-, and *Mlu*I (*slots 10, 11,* and *12*)-cleaved λDNA was incubated with HB8 DNA ligase (1.73 units, *slots 2, 3, 5, 6, 8, 9, 11,* and *12*) at 24 °C overnight. *Slots 3, 6, 9,* and *12* show recutting by *Hin*dIII-, *Pst*I-, *Eco*RI-, and *Mlu*I-endonucleases of the products formed by ligation, respectively. The ligation products were isolated by phenol extraction and ethanol precipitation. The precipitates were dissolved in the reaction mixtures of the respective restriction enzymes, and incubated with the respective enzymes (6 units) at 37 °C for 2 h. These products were analyzed by 0.9% agarose gel electrophoresis. Detailed conditions are described under "Methods." *LD*, ligation with HB8 DNA ligase; *RC*, recutting.

The most significant finding for this enzyme was its temperature dependency of ligation and the thermostability. Optimum temperatures of ligation of HB8 DNA ligase on various substrates were compared with those of T4 DNA ligase under the same conditions. The temperature optimum of HB8 DNA ligase was found to shift to a region 7–10 °C higher than that of T4 DNA ligase for any substrate. The optimum temperatures of ligation with *E. coli* and T4 DNA ligases have been reported for various substrates (21–25) and agree with our data. In 1972 Harvey and Wright (21) reported on the joining of thymidylate oligomers by T4 DNA ligase and for each oligomer length, there is a distinct optimum temperature at which joining takes place most readily. They found the optimum temperature of $p(dT)_8$ with poly(dA) to be 17.5 °C and that of $p(dT)_{13}$ with poly(dA), 32.5 °C. In the case of restriction enzyme-cleaved DNA, the optimum ligation temperature is somewhat confusing. Dugaiczyk *et al.* (22) reported that the optimum temperature for the covalent joining of cohesive termini of DNA ranges from 10 to 15 °C in the case of *E. coli* DNA ligase. The temperature optimum of T4 DNA ligase for both cohesive- and blunt-end joinings has been reported to be about 25 °C by electron microscopic assay (23). Ferretti and Sgaramella (24) have revised their previous results and indicated that joining was maximal at 4 °C and decreased with increasing temperatures in the manner of sigmoid-like curves. The reason for this may possibly be that contamination of the ligase preparation may have reduced the extent of joining at low temperatures. For conversion of nicked circular DNA to closed circular DNA, the optimum temperature of T4 DNA ligase has been reported to be around 37 °C (25). The joining optimum is affected not only by individual melting temperatures of various substrates, but by conformation of

DNA ligase itself. Usually, the optimum temperature of a substrate is higher than its melting temperature. At the temperature at which the joining rate is maximal, the physical polymer-oligomer complex should not be stable and in the presence of ligase, a stable complex formed (21). It is quite reasonable that HB8 DNA ligase has nick-closing activity up to 85 °C, considering that cells can grow from 60 to 85 °C and activity possibly reflects the role of the enzyme *in vivo* as in DNA replication and DNA repair. HB8 DNA ligase showed little activity around 4 °C in contrast with T4 DNA ligase. This suggests that HB8 DNA ligase takes on an inactive conformation at low temperatures.

The thermostability of HB8 DNA ligase was confirmed by its being stable for 1 week at 37 °C without loss of activity. The enzyme could be stored for several months at −20 °C and could withstand freezing and thawing in 20% glycerol.

Up to now, only T4 DNA ligase has been known to be capable of catalyzing the joining of duplexes at fully base paired ends, *i.e.* blunt-end joinings. The *E. coli* enzyme is totally inactive in this reaction (4, 26). In fact, on the basis of the present data, a blunt-end joining has not been shown to occur through the action of HB8 DNA ligase. It is known that blunt-end joinings require large amounts of enzyme, in contrast to that required for the joining of cohesive fragments (26). In our case, the enzyme concentration was not sufficiently high to detect the blunt-end ligation, being only about one-hundredth of that of commercially available T4 DNA ligase. More concentrated enzyme solutions will be necessary for further investigation to confirm blunt-end ligation by HB8 DNA ligase. Recently DNA ligase preparations from rat liver nuclei or from *E. coli* have been reported to actively catalyze the blunt-end ligation of DNA in the presence of high concentrations of various nonspecific polymers (27) and the rates of blunt-end and cohesive-end ligation of DNA by T4 DNA ligase increase greatly in the presence of macromolecules (28). Blunt-end ligation by HB8 DNA ligase should be investigated under these conditions.

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Supplement to: Thermophilic DNA Ligase. Purification and Properties of the Enzyme from Thermus Thermophilus HB8 by Miho Takahashi, Eiichi Yamaguchi and Tsuneko Uchida

EXPERIMENTAL PROCEDURES

Materials — [γ-^{32}P]ATP was purchased from the Biochemical Center (Amersham, England). Oligothymidylate p(dT)$_8$, p(dT)$_{18}$, p(dT)$_{12-18}$, p(dG)$_{12-18}$, p(dA)$_{12-18}$, poly(dA), poly(dT) and poly(dC) were obtained from Collaborative Research Inc., λDNA, from Bethesda Research Laboratories, PM2 DNA, polynucleotide kinase, T4 DNA ligase and PstI endonuclease from Boehringer Mannheim GmbH. Alkaline phosphatase was purchased from Sigma Chemical Co., DNaseI from Worthington Biochemical Co. Hind III, Hlu I and Eco RI endonucleases from Takara Shuso Inc. DNA-cellulose was obtained from Miles Laboratories, Inc., Sepharose 6B from Pharmacia Fine Chemicals, DE52 and P11 from Whatman Ltd., and Carrier ampholites for isoelectric focusing were products of LKB-Produckter AB. Polyethylene glycol (# 20,000) was purchased from Nakarai Chemicals, LTD., Kyoto.

Methods

Preparation of 5'-^{32}P-Labeled Oligomers: Deoxyoligomers were dephosphorylated and labeled with ^{32}P in the 5' position with polynucleotide kinase, as described by Harvey et al (9) with modifications (10). The oligomers were dephosphorylated in a reaction mixture (200 µl) containing 2 A_{260} units of oligomer, 0.1 M ammonium bicarbonate, pH 8.0 and 20 µg of E. coli alkaline phosphatase. After incubation for 2 h at 37°C, the mixture was autoclaved for 5 min at 121°C, 1.23–1.38 Kg/cm^2 to inactivate the enzyme. The mixture was diluted with 1 ml of distilled water and lyophilized. The reaction mixture (1.8 ml) containing 0.78 A_{260} units of 5'-OH oligomers, 40 mM ammonium bicarbonate, 10 mM $MgCl_2$ and 2 mM spermine was boiled for 2 min, cooled quickly, and 8 mM dithiothreitol, 1 nmole of [γ-^{32}P]ATP (200 µCi) and 12 units of polynucleotide kinase were added to make up 2 ml. The reaction mixture was incubated for 3 h at 37°C. After the reaction was stopped by boiling for 2 min, the reaction mixture was charged on Sephadex G-50 column (1.2 × 95 cm) equilibrated with 50 mM triethylamine buffer, pH 7.6 to remove remaining [γ-^{32}P]ATP. The product was collected, lyophilized and dissolved in 10 mM Tris-HCl, pH 7.6 containing 1 mM EDTA.

Preparation of Nicked DNA: Nicked DNA was prepared with pancreatic DNase I in the presence of a saturating amount of ethidium bromide according to the method of Barzilai (11). The reaction mixture (5 ml) contained 10 mM Tris, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mg/ml of bovine serum albumine, PM2 DNA (0.5 A_{260}) and 3 µg of ethidium Bromide/µg of DNA. After 10 Ug of DNase I was added, the reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by adding the same volume of phenol saturated with 10 mM Tris-HCl, pH 8.0. The upper layer was precipitate with ethanol. The precipitate was dissolved in 10 mM Tris-HCl, pH 7.6, containing 1 mM EDTA and served as nicked DNA.

Preparation of Restriction Endonuclease - cleaved λDNA: Digestion of λDNA with various restriction endonucleases were done under the conditions by the suppliers. The digested DNA was isolated by phenol extraction and ethanol precipitation.

Assay for DNA Ligase: DNA ligase was assayed by the method of Rase et al (12) with modifications. The reaction mixture (40 µl) contained 50 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 6.6 mM dithiothreitol, 66 µM NAD, 400 pmole poly(dA), 400 pmole [5'-^{32}P](dT)$_{18}$ (concentration in total phosphate) and the enzyme. After incubation at 37°C for 30 min, the reaction was terminated by heating at 100°C for 3 min. To the reaction mixture 5 µl of alkaline phosphatase (0.1 units) were added and the mixture was incubated at 65°C for 30 min. After the addition of 50 µl of carrier DNA (2 mg/ml), the mixture was spotted on a Whatman GF/F glass fiber disk (2.4 cm diameter) and the disk was washed in cold 5% CCl_3COOH containing 1% NaPPi for 20 min twice, succesdingly in cold 5% CCl_3COOH for 20 min twice, in cold ethanol and dried in ether. The radioactivity of the acid-insoluble materials on the disk was counted in toluene scintillator. One unit of the enzyme is defined as being equivalent to 1 nmol of ^{32}Pi resistant to alkaline phosphatase under standard assay conditions. One unit of A_{260} is the quantity of protein which has an absorbance of 1.0 when dissolved in 1 ml of buffer A and measured in a 1 cm light path at 260 nm.

Assay for Nick-closing Activity: For nick-closing activity, 30 µl of reaction mixtures containing 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.6 mM NAD, ca. 0.25 µg of nicked DNA (form II PM2 DNA) and 0.5 µl of purified HB8 DNA ligase were incubated at various temperatures. The reaction was terminated by the addition of 15 µl of stop solution consisted of 20 mM EDTA, 60% sucrose and 0.01% bromphenol blue and the sample was applied to a 0.7% agarose slab gel.

Cohesive-end and Blunt-end Ligation of DNA Fragments: DNA ligase activity was also assayed for its ability to ligate cohesive- and blunt-end DNA fragments with restriction endonucleases. For ligation assay, 30 µl reaction mixtures containing 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.6 mM NAD, 0.5 µg of restriction endonuclease-digested λDNA, and 1-2 µl of purified HB8 DNA ligase were incubated at 24°C overnight. The reaction was terminated by the addition of 15 µl stop solution consisted of 20 mM EDTA, 60% sucrose and 0.01% bromphenol blue and the samples were applied to a 0.9% agarose slab gel.

Nondenaturing Polyacrylamide Gel Electrophoresis: Electrophoresis was carried out in 7.5% acrylamide gels at pH 8.9 as described by Orstein (13) and Davis (14). The purified DNA ligase (3.8 µl) was loaded on gels (0.6 x 11.4 cm) with bromphenol blue. After electrophoresis (at 6 mA/tube for 4 h), one gel was stained with Coomassie brilliant blue. The other gel was cut into 1 mm slices so as to extract the ensyme with 200 µl of buffer A. The DNA ligase activity was measured with 10 µl of the extract for 4 h at 37°C.

SDS-Polyacrylamide Gel Electrophoresis: SDS-7.5% polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn (15) with a constant current of 25 mA/slab (14 x 16 x 0.15 cm) at room temperature for 5 h. The gel was stained first with Coomassie brilliant blue and destained in 40% methanol/10% acetic acid to silver with a Bio-Rad silver stain kit succeedingly. The marker proteins used were phosphorylase b (Mr=94,000), albumin (Mr=67,000) ovalbumin (Mr=43,000), carbonicanhydrase (Mr=30,000), trypsin inhibitor (Mr=20,100) and α-lactalbumin (Mr=14,400).

8M Urea-Polyacrylamide Gel Electrophoresis: The reaction products of [5'-^{32}P](dT)$_{18}$ with poly(dA) were analyzed by electrophoresis in Tris-borate-EDTA, pH 8.3, on 20% polyacrylamide containing 8 M urea (16). The samples containing 8 M urea and 0.01% of xylene cyanol and bromphenol blue were loaded on a vertical slab gel (14 x 16 x 0.15 cm) at 400V for 3 h. After the electrophoresis, the gel was autoradiographed on a Kodak-o-Mat RP16 film at -80°C.

Agarose Gel Electrophoresis: The ligation products of DNA fragments with restriction endonucleases or the products of nicked DNA were analyzed by agarose gel electrophoresis. The electrophoresis was carried out in Tris-Acetic acid-EDTA, pH 8.0, containing ethidium bromide (5 ng/ml) on 0.7% or 0.9% agarose vertical slabs (14 x 16 x 0.15 cm) at 40 mA for 3 h (17). The gels were photographed under UV light.

Purification of DNA ligase

All procedures were carried out at 4°C.

Preparation of the Crude Extract —— T. thermophilus HB8 (=ATCC 27634) was kindly donated by Dr. T. Oshima of Tokyo Institute of Technology. The cells were grown at 75°C in a medium of 0.8% polypeptone (Kyokuto Seiyaku Co., Osaka), 0.4% yeast extract (Difco Laboratories, Detroit), 0.2% NaCl and 0.05% basal elements (pH 7.0) (19), and harvested at the logarithmic phase (ca. 2 x 10^9/ml). The frozen cells (1.6 Kg) were mixed with 7 liters of 0.02 M Tris-HCl, pH 7.6, containing 1 mM 2-mercaptoethanol and 0.1 mM EDTA with a Waring blender. The mixture was further homogenized with a Dyno-mill (Type KDL, Willy A. Bachofen Manufacturing Engineers, Switzerland) containing 0.1 mm glass beads and centrifuged at 9,000 rpm for 15 min. The supernatant was served as a crude extract.

Streptomycin and Ammonium Sulfate Fractionation —— To the crude extract, a 5% streptomycin solution was added to make final concentration at 1%. The mixture was centrifuged at 9,500 rpm for 20 min and to the supernatant, solid ammonium sulfate was added to 50% saturation. After centrifuging the supernatant at 9,000 rpm for 15 min, the resulting precipitate was dissolved in 10 mM KH_2PO_4 -Na_2HPO_4, pH 7.6, containing 0.1 M KCl and 20% glycerol and dialyzed against the same buffer.

Phosphocellulose (P11) Column Chromatography —— The dialyzed solution, 1.6 liters, was applied to a P11 column (8.6 x 64 cm) equilibrated with the above buffer. After the column was washed with 4.6 liters of the same buffer, the elution was performed with a linear KCl gradient (0.1 M to 0.5 M) in 5 liters of the phosphate buffer. Enzyme activity was detected in the region of 0.15-0.21 M KCl concentration and the active fractions (25 ml/fraction) were combined. The combined solution (1 liter) was placed in Visking cellulose tubing and immersed in 3 liters of 30% polyethylene glycol (# 20,000) dissolved in 20 mM Tris-HCl, pH 7.6, containing 0.1 M KCl and 20% glycerol (bufer A). When the volume of the enzyme solution inside of the dialysis tubing was reduced to about 100 ml, the dialysis buffer was changed to buffer A. Unless otherwise noted, the enzyme solution was concentrated with this method through the following purification procedures.

DEAE-cellulose (DE52) Column Chromatography —— The concentrated enzyme solution (87 ml) was further dialyzed against buffer A and applied to a DE52 column (3.6 x 41 cm) equilibrated with the same buffer. The enzyme passed immediately from the column, without adsorbing at all to DE52. The fractions were then combined (240 ml) and concentrated in the manner indicated above.

DNA-cellulose Column Chromatography —— To the concentrated enzyme solution (20 ml), NMN and $MgCl_2$ were added to a final concentrations of 7 mM and 5 mM respectively. The mixture was kept at 25°C for 30 min to release AMP from the enzyme-adenylate complex. After stopping the reaction by the addition of 10 mM EDTA, the mixture was dialyzed against buffer A. The dialysate was divided into 4 parts and applied to a DNA-cellulose column (1.2 x 9 cm) equilibrated with buffer A containing 10 mM $MgCl_2$. Some of the activity appeared as a small peak situated behind that of the main protein of the break through fractions and the most of the enzyme had been eluted with 0.25 M KCl in buffer A. The combined active fractions were concentrated to 23 ml.

Sepharose 6B Column Chromatography —— The concentrated enzyme solution was divided into two fractions and applied to a Sepharose 6B column (1.8 x 124 cm) equilibrated with buffer A. Fractions of 40 drops each were collected and enzyme activity was detected between fractions 110 and 114. The active fractions were combined and concentrated.

Isoelectric Focusing Column Chromatography —— On the concentrated enzyme solution (14.8 ml), isoelectric focusing column chromatography (440 ml) was carried out at pH 5-7 with Ampholine at a final concentration of 2% at 300V for 62 h. The enzyme activity appeared around pH 6.6.

The concentrated enzyme solution (7.5 ml) was once more applied to the Sepharose 6B column to remove any remaining Ampholite. The active fractions were collected, concentrated and used as a purified HB8 DNA ligase. The overall purification is summarized in Table I. In purification steps 1 and 2, no DNA ligase activity could be detected, because of contamination from nucleases and phosphomonoesterases. The final yield of the purified DNA ligase had 14% of the activity in step 3.

Table I

Purification of HB8 DNA ligase from Thermus thermophilus HB8

Enzyme activity was assayed as described under "Method".

Purification step	Vol.	Protein	Activity	Total activity	Yield	Specific activity
	ml	A_{280}/ml	Units/ml	units	(%)	units/A_{280}
1) Crude Extract	7,200	63.3	-	-		-
2) $(NH_4)_2SO_4$(<50%)	1,590	27.8	-	-		-
3) Phosphocellulose(P 11)	87	11.5	420	36,500	(100)	36
4) DEAE-cellulose(DE 52)	28	26.5	790	22,120	(61)	30
5) DNA-Cellulose	23	0.91	750	17,250	(47)	824
6) Sepharose 6B (1)	14.8	0.54	1,077	15,940	(44)	1,994
7) Ampholine E. F.	7.5	0.62	1,416	10,624	(29)	2,285
8) Sepharose 6B (2)	3.0	0.82	1,729	5,180	(14)	2,106

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A Ligase-Mediated Gene Detection Technique

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An assay for the presence of given DNA sequences has been developed, based on the ability of two oligonucleotides to anneal immediately adjacent to each other on a complementary target DNA molecule. The two oligonucleotides are then joined covalently by the action of a DNA ligase, provided that the nucleotides at the junction are correctly base-paired. Thus single nucleotide substitutions can be distinguished. This strategy permits the rapid and standardized identification of single-copy gene sequences in genomic DNA.

DNA ANALYSIS IS ATTAINING increasing importance for the diagnosis of disease caused by single-gene defects as well as for the detection of infectious organisms (*1*). Moreover, a number of genes, predominantly those encoded in the major histocompatibility complex, have been found to be associated with an increased susceptibility to a variety of disease states (*2*). Of a total of approximately 2000 defined human genetic loci (*3*), approximately 100 have currently been studied at the DNA level for their role in genetic disease (*4*). A number of genetic diseases are caused by alleles present in the population at relatively high frequencies, perhaps because of selective advantages to the heterozygous carriers (*5*). The ongoing characterization of disease-causing or disease-associated gene sequences makes large-scale screening for carrier status and genetic counseling a possibility. It may also sharpen the diagnostic accuracy for diseases such as autoimmune conditions where the susceptibility may be influenced by defined alleles. Such prospects are currently limited by the cumbersome nature of the available DNA detection methods.

A majority of polymorphisms in the human genome are caused by point mutations that involve one or a few nucleotides. Current DNA analysis procedures capable of detecting the substitution of a single nucleotide are based on differential denaturation of mismatched probes as in allele-specific oligonucleotide hybridization (*6*) or denaturing gradient gel electrophoresis (*7*). Alternatively, the sequence of interest can be investigated for polymorphisms that affect the recognition by a restriction enzyme (*8*) or that will allow ribonuclease A (RNase A) to cleave at mismatched nucleotides of an RNA probe hybridized to a target DNA molecule (*9*). Although denaturing gradient gel or RNase A can survey long stretches of DNA for mismatched nucleotides, they are estimated to detect only about half of all mutations that involve single nucleotides (*7*, *9*). Similarly, less than half of all point mutations give rise to gain or loss of a restriction enzyme cleavage site (*10*). The only existing technique capable of identifying any single nucleotide difference, short of DNA sequence analysis, is allele-specific oligonucleotide hybridization. This technique involves immobilizing separated (*6*) or enzymatically amplified (*11*) fragments of target DNA, hybridizing with oligonucleotide probes, and washing under carefully controlled conditions to discriminate single nucleotide mismatches.

We have devised a strategy that permits the facile distinction of known sequence variants differing by as little as a single nucleotide. The approach combines the ability of oligonucleotides to hybridize to the sequence of interest and the potential of a DNA-specific enzyme, T4 DNA ligase, to distinguish mismatched nucleotides in a DNA double helix (Fig. 1). Two oligonucleotide probes are permitted to hybridize to the denatured target DNA such that the 3′ end of one oligonucleotide is immediately adjacent to the 5′ end of the other. The ligase can then join the two juxtaposed oligonucleotides by the formation of a phosphodiester bond, provided that the nucleotides at the junction are correctly base-paired with the target strand. The ligation event thus positively identifies sequences complementary to the two oligonucleotides. A heterozygous sample is therefore scored as positive for both alleles. The joining of the oligonucleotides may be conveniently demonstrated, for instance, by labeling one of the oligonucleotides with biotin and the other one with ^{32}P. After the ligation reaction, the biotinylated oligonucleotides are allowed to bind to streptavidin immobilized on a solid support. Radioactive oligonucleotides that have become ligated to biotinylated oligonucleotides remain on the support after washing and are detected by autoradiography.

The gene encoding human β globin was selected as a model system to test the technique. There are two relatively frequent alleles, β^S and β^C, each differing from the normal allele, β^A, by a single nucleotide substitution in positions 2 and 1, respectively, of codon six (Figs. 2 and 3) (*12*). Subjects homozygous for the β^S allele suffer from sickle cell anemia. Moreover an increased risk of sudden death during exertion has been observed among individuals heterozygous for β^S (*13*).

The ligase-mediated gene detection procedure was used to distinguish β^A and β^S genes in equivalent amounts of DNA present in cells, in cloned DNA, and in genomic DNA (Fig. 2). One of two synthetic oligonucleotides (B131 or B132), specific for each of the alleles, was used in conjunction

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with another oligonucleotide (P133) hybridizing immediately 3′ to either of the other two oligonucleotides on the target DNA strand. All of the synthetic oligomers used in this study are 20 nucleotides long. The ability of T4 DNA ligase to join the variable, 3′ nucleotide of the allele-specific oligonucleotides to the 5′ terminus of the invariant oligonucleotide was assessed by capturing any ligated product on streptavidin-agarose beads. The beads were filtered and washed to remove unbound oligonucleotides, and then the filter with trapped beads was exposed to x-ray film. The 10^6 nucleated cells used for one assay were obtained from ~0.5 ml of blood. The cells were used in the assay without DNA purification, by first making the DNA accessible for the ligase-mediated analysis by sequential additions of a nonionic detergent (Triton X-100) and a protease (trypsin). The DNA was denatured with alkali and then soybean trypsin inhibitor was added to prevent proteolysis of the added ligase.

The described ligation reactions were performed at 37°C, ~25 K below the melting temperature of the hybridized oligonucleotides, permitting the use of standardized assay conditions independent of the particular sequence investigated. The observed specificity is a consequence of the requirement for the simultaneous hybridization of both oligonucleotides in a precisely juxtaposed position. Although both oligonucleotides are likely to hybridize to numerous sequences in the DNA sample, they are unlikely to do so in the appropriate head-to-tail fashion except where the proper target sequence is present. In addition, we have found that the ligation reaction requires that the two terminal nucleotides on either side of the junction of the two oligonucleotides be engaged in correct base-pairing. This requirement further suppresses incorrect ligation events.

To determine whether any type of single nucleotide mismatch could be distinguished from correct base-pairing with the present method, we used four synthetic target molecules representing a segment of the β-globin gene, each with a different nucleotide in the first position of the sixth codon. Two of the sequences are derived from the β^A and β^C alleles of the β-globin gene. The other two sequences represent the other possible nucleotides occupying the variant position. Four pairs of oligonucleotides were designed to specifically identify one of the target molecules. Four oligonucleotide probes, each with a different nucleotide in the 3′ terminal position and complementary to one of the target molecules, were separately assayed for their ability to be ligated to an invariant oligonucleotide that hybrid-

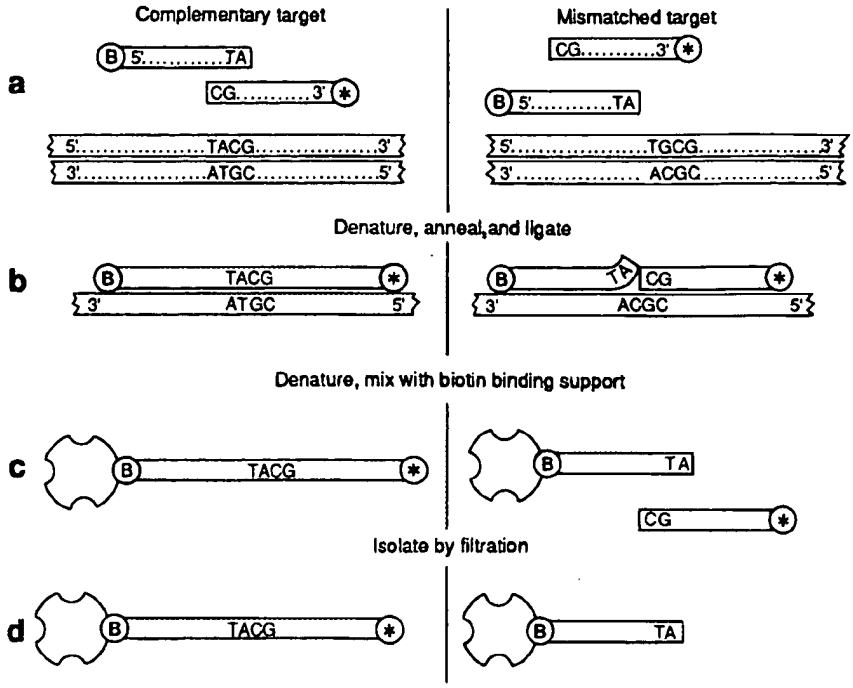

Fig. 1. A diagram depicting gene detection through the ligation of hybridized oligonucleotide probes. Target DNA is denatured and mixed with oligonucleotides and ligase. The ligase joins pairs of oligonucleotides annealed head to tail if they are correctly base-paired at the junction. Radioactively labeled oligonucleotides (*) are immobilized and detected by autoradiography only if ligated to biotinylated oligonucleotides (B) that can be bound to streptavidin on a solid support.

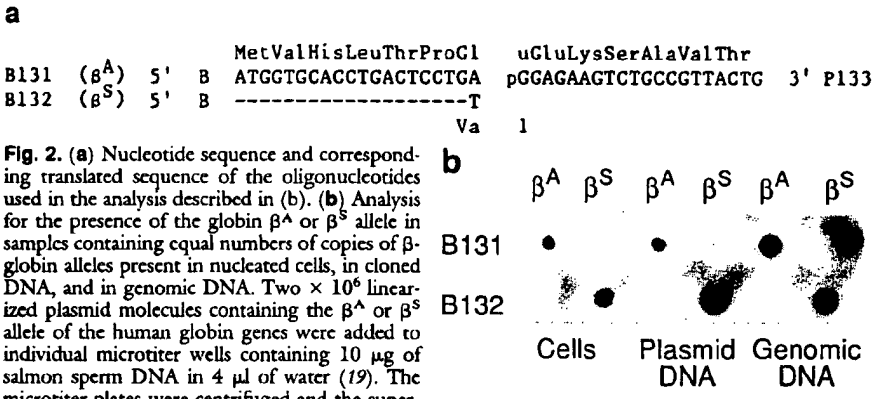

Fig. 2. (**a**) Nucleotide sequence and corresponding translated sequence of the oligonucleotides used in the analysis described in (b). (**b**) Analysis for the presence of the globin β^A or β^S allele in samples containing equal numbers of copies of β-globin alleles present in nucleated cells, in cloned DNA, and in genomic DNA. Two × 10^6 linearized plasmid molecules containing the β^A or β^S allele of the human globin genes were added to individual microtiter wells containing 10 μg of salmon sperm DNA in 4 μl of water (*19*). The microtiter plates were centrifuged and the supernatants removed. To the resuspended cell pellet was added 1 μl of 10% Triton X-100 and 1 μl of trypsin at 2 μg/μl. The samples were incubated at 37°C for 30 min and were denatured with alkali as above. The *p*H was neutralized and 1 μl of soybean trypsin inhibitor (Sigma, 10 μg/μl) was added. Each well received 140 fmol of biotinylated oligonucleotides B131 or B132 (*20*), specific for the globin β^A and β^S genes, respectively, and 1.4 fmol of oligonucleotide P133, 5′ end-labeled with [γ-^{32}P]adenosine triphosphate (ATP) and polynucleotide kinase to a specific activity of 5 × 10^8 Cerenkov cpm/μg and purified over a Nensorb column (Du Pont Biotechnology Systems). T4 DNA ligase (0.05 Weiss unit, Collaborative Research) was added in 2 μl of 5× ligase buffer to a final volume of 10 μl containing 50 m*M* tris-HCl (*p*H 7.5), 10 m*M* $MgCl_2$, 150 m*M* NaCl (including 50 m*M* added during denaturation), 1 m*M* spermidine, 1 m*M* ATP, 5 m*M* dithiothreitol, and 100 ng of bovine serum albumin per microliter. The reagents were mixed by briefly centrifuging the microtiter plates before incubating at 37°C and 100% humidity for 5 hours. The ligated oligonucleotides were denatured by the addition of 1 μl of 1.1*M* NaOH and incubated for 10 min at 37°C. After the incubation, 1 μl of 1.1*M* HCl and 2 μl of 10% SDS were added. Three microliters of a 15% (v/v) suspension of streptavidin-coated agarose beads (Bethesda Research Laboratories) was then added, and the plate was incubated on a shaking platform at room temperature for 5 min. The contents of the wells were transferred to a dot blot manifold (Schleicher and Schuell) with a Whatman filter paper no. 4. In order to reduce nonspecific binding of the labeled oligonucleotides, the filter papers had been boiled and the beads diluted in 0.5% (v/v) dry nonfat milk, 1% SDS, and salmon sperm DNA (100 μg/ml). The beads (*21*) were washed under suction in the manifold with 3 ml of 1% SDS and 1 ml of 0.1*M* NaOH per sample, with a 96-tip dispenser (Vaccu-pette/96, Culture Tek). The filters were wrapped in plastic wrap and autoradiographed for 3 days at −70°C with one enhancing screen (Du Pont).

ized immediately 3′ to the first oligonucleotide. These reagents permit studying the effect on ligation by any of the 16 possible base pairs, the 4 correct Watson-Crick pairs and 12 mismatched pairs, in an invariant sequence context. Under the appropriate conditions, only nucleotides engaged in correct base-pairing were efficiently joined by ligation (Fig. 3). Parameters that affected the nucleotide specificity were the salt concentration and the amount of enzyme added relative to the DNA concentration. Higher salt concentration and lesser amounts of enzyme than those found to be optimal for discrimination resulted in loss of signal. The above experiment cannot exclude the possibility that the identification of mismatched nucleotides may be influenced by the surrounding sequence, although we have not yet encountered any evidence for such effects.

Although autoradiographic techniques are relatively simple to implement, a gene detection assay based on the use of fluorescent rather than radioactive probes would have the advantages of safe handling, more stable reagents, and rapid access to the results, and would allow for multicolor analysis by using fluorophores with different emission spectra. In general, conventional organic fluorophores are less sensitive labels than ^{32}P. Thus we increased the amount of target DNA before the detection assay with the polymerase chain reaction (*14*). With this procedure a segment of DNA can be exponentially amplified by repeated cycles of enzymatic synthesis of new strands from two oligonucleotide primers, one with a sequence derived upstream and the other in the opposite orientation downstream of the segment of interest. Genomic DNA was obtained from three human cell lines, MOLT-4, which is homozygous for the β^A-globin allele; SC-1, homozygous for the β^S allele; and GM2064, in which the β-globin locus has been deleted (*15*). The appropriate segment of the β-globin gene was amplified in 25 cycles from 1 μg of genomic DNA from each cell line. We used 3-μl aliquots, equivalent to 24 ng of genomic DNA for the assay. Two oligonucleotides, specific for the β^A and β^S alleles and differentially 5′-labeled with one of two fluorophores, were present at equal concentrations. The amount of each of these oligonucleotides that became ligated to a third oligonucleotide hybridizing downstream of the other two was determined by separating the reaction products on an 8% polyacrylamide gel and analyzing the band migrating as a 40-nucleotide oligomer (the size of two ligated oligonucleotides) for the relative contribution by the two different fluorophores [model 370A DNA sequencer, Applied Biosystems, Foster City, California (*16*)]. No signal was observed when the β-globin gene had been deleted in the cell from which the DNA was obtained, whereas only the correct fluorophore-labeled oligonucleotide was ligated when the cells harbored the β^A or β^S alleles

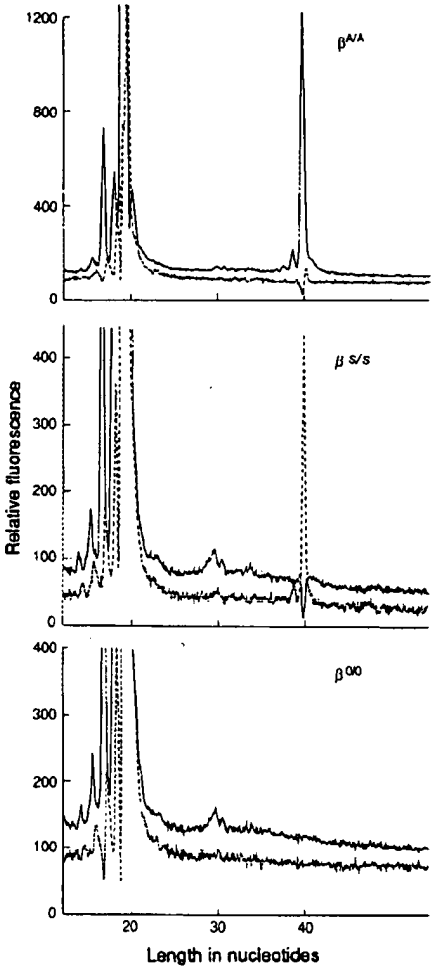

Fig. 4. Demonstration of the presence of the β^A and β^S alleles of the β-globin gene in amplified genomic DNA by probes labeled with fluorescent dyes. A 120-bp segment of the β-globin gene was amplified with the polymerase chain reaction as described (*16*) in 25 cycles starting with 1 μg of genomic DNA from the cell lines MOLT-4, SC-1, and GM2064 ($\beta^{A/A}$, $\beta^{S/S}$, and $\beta^{0/0}$, respectively) in 100 μl. Three microliters of each amplified sample was added to an Eppendorf tube, denatured by alkali, neutralized, and incubated with 14 fmol each of oligonucleotide 131 labeled with carboxy-fluorescein (Molecular Probes) (CF131) (——) and oligonucleotide 132 labeled with carboxy-2′,7′-dimethoxy-4′,6′-dichlorofluorescein (CD132) (– – –), and 14 fmol of nonradioactively 5′ phosphorylated oligonucleotide P133 (for sequences, see Fig. 2). The reaction conditions were essentially as described in Fig. 2, but 0.5 Weiss unit of T4 DNA ligase was added to each assay. At the end of the 3-hour incubation, the samples were ethanol precipitated, taken up in 50% formamide, and loaded on a sequencing gel in an ABI 370A automated DNA sequencer. The fluorescence signal was processed to distinguish the partially overlapping emission spectra of the two fluorophores and to determine the relative contribution of each fluorophore to the signal.

a

```
B128  (βA)  5' B  CATGGTGCACCTGACTCCTG  pAGGAGAAGTCTGCCGTTACT 3' P129
B134  (βC)  5' B  -------------------A
B136        5' B  -------------------T
B137        5' B  -------------------C

172   (βA)  3'    GTACCACGTGGACTGAGGACTCCTCTTCAGACGGCAATGA   5'
138   (βC)  3'    -------------------T--------------------   5'
139         3'    -------------------A--------------------   5'
140         3'    -------------------G--------------------   5'
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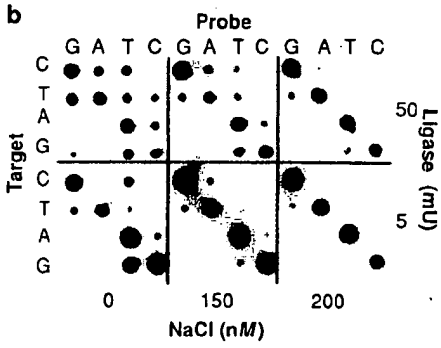

Fig. 3. (**a**) Nucleotide sequence of the oligonucleotides used in the analysis described in (b). (**b**) Correct identification of four target molecules, differing by single-nucleotide substitutions in one position. Letters refer to the variable nucleotides in the probe and target sequences. As target molecules, 40-nucleotide oligomers, derived from the β-globin gene sequence, were synthesized. The oligonucleotides 172, 138, 139, and 140 are of identical sequence except in a central position where each target molecule includes a different nucleotide. Four 20-nucleotide biotinylated oligomers, B128, B134, B136, and B137, differing only in their 3′ nucleotide position, were designed to hybridize to the 3′ half of the target molecules such that the variant position of the probe reagents corresponds to that of the target molecules. Each of the biotinylated oligonucleotides was used in conjunction with oligonucleotide P129, 5′ end-labeled with ^{32}P and hybridizing immediately 3′ to the biotinylated probes on the target strands. The assays were performed essentially as described in the legend to Fig. 2, but 2×10^8 copies of one of the target molecules were added to each well with 10 μg of salmon sperm DNA. Each well further received one of the biotinylated oligonucleotides together with oligonucleotide P129. The final NaCl and ligase concentrations were varied as indicated.

(Fig. 4). This strategy could be generalized to the simultaneous analysis of several loci. For each set of two labeled, allele-specific oligonucleotides and one unlabeled, the latter is given a nonhybridizing 3′ sequence extension of a unique length. This results in different migration rates for the ligation products, characteristic of each locus.

In contrast to gene detection techniques based on immobilizing the target DNA, such as DNA blots, the hybridization reported here was performed in solution and in a small volume, which reduced the time required for hybridization (*17*). It also obviated the step of immobilizing the target DNA. Both ligation and binding of the biotinylated oligonucleotides are efficient and rapid steps that should permit quantitative detection of target molecules. In general, there are three rate-limiting steps in gene detection techniques. The first is sample preparation, which can be greatly simplified as demonstrated here. The second is the time required for the probes to anneal to the target sequence. This is a function of the concentration of the probe and can be reduced considerably. The third and most time-consuming step in the present technique is signal detection by autoradiography. A sufficiently sensitive fluorescent detection method (*18*) should drastically reduce this time, permitting the development of a rapid, automated gene detection procedure.

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19. Genomic DNA was purified from guanidinium HCl–solubilized cells as described [D. Bowtell, *Anal. Biochem.* **162**, 463 (1987)] and resuspended by boiling before adding 7 μg in 4 μl of water per assay well. The plasmid and genomic DNA samples were denatured by adding 1 μl of 0.5*M* NaOH and incubating for 10 min at 37°C before restoring the *p*H with 1 μl of 0.5*M* HCl. Alternatively, samples of nucleated blood cells were used directly as a source of DNA for the analysis. Cells (10^6), obtained by Ficoll-Hypaque (Pharmacia) flotation, were added in 50 μl of phosphate-buffered saline to each well.
20. The oligonucleotides were assembled by the phosphoramidite method [S. J. Horvath, J. R. Firca, T. Hunkapiller, M. W. Hunkapiller, L. Hood, *Methods Enzymol.* **154**, 314 (1987)] on an Applied Biosystems model 380A DNA synthesizer and purified either by polyacrylamide gel electrophoresis or reversed-phase high-pressure liquid chromatography (HPLC). Biotinylation was performed by reacting a biotin *N*-hydroxysuccinimide ester (Enzotin, Enzo) with a 5′ aminothymidine residue incorporated in the oligonucleotide [L. M. Smith, S. Fung, T. J. Hunkapiller, M. W. Hunkapiller, L. Hood, *Nucleic Acids Res.* **13**, 2399 (1985)]. The product was purified by reversed-phase HPLC.
21. The size of the area on which the beads were deposited was reduced by interposing a 3-mm-thick plexiglass disk with conical holes with diameters of 5 mm on the upper surface and 2 mm on the lower.

22. The authors acknowledge a stipend from the Knut and Alice Wallenberg Foundation to U.L. and support from NSF grant BNS 87 14486, Defense Advanced Research Projects Agency grant N00014-86K-0755, Upjohn Company, and Applied Biosystems, Inc. The oligonucleotides were synthesized by S. J. Horvath and the fluorescence data were analyzed by C. Dodd. R. K. Saiki provided plasmids and samples of genomic DNA obtained from cell lines. J. Korenberg and K. Tanaka made available blood samples from sickle cells patients. The *N*-hydroxysuccinimide ester of carboxy-2′,7′-dimethoxy-4′,5′-dichlorofluorescein was provided by M. W. Hunkapiller. We acknowledge discussions with B. Korber, B. Popko, A. Kamb, N. Lan, L. Smith, R. Barth, V. A. McKusick, J. Richards, and M. Simon.

11 April 1988; accepted 23 June 1988

Amyloid Protein Precursor Messenger RNAs: Differential Expression in Alzheimer's Disease

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In situ hybridization was used to assess total amyloid protein precursor (APP) messenger RNA and the subset of APP mRNA containing the Kunitz protease inhibitor (KPI) insert in 11 Alzheimer's disease (AD) and 7 control brains. In AD, a significant twofold increase was observed in total APP mRNA in nucleus basalis and locus ceruleus neurons but not in hippocampal subicular neurons, neurons of the basis pontis, or occipital cortical neurons. The increase in total APP mRNA in locus ceruleus and nucleus basalis neurons was due exclusively to an increase in APP mRNA lacking the KPI domain. These findings suggest that increased production of APP lacking the KPI domain in nucleus basalis and locus ceruleus neurons may play an important role in the deposition of cerebral amyloid that occurs in AD.

ALZHEIMER'S DISEASE (AD) IS characterized pathologically by large numbers of senile plaques and neurofibrillary tangles throughout the cerebral cortex and hippocampus. Senile plaques consist of clusters of degenerating neurites surrounding an amyloid core composed of 5- to 10-nm fibrils that stain metachromatically with Congo red. In many cases of AD, amyloid fibrils are also found in vessel walls (*1*). A 4.2-kD polypeptide, referred to as A4 or the β protein, has been isolated from the amyloid fibrils found in senile plaques (*2*) and vessel walls (*3*) of patients with AD. There is evidence that A4 may also be a component of the paired helical filaments found in neurofibrillary tangles (*4*).

The gene encoding A4, which is located on chromosome 21 (*5*), produces at least three mRNAs (Fig. 1) referred to as APP_{695}, APP_{751}, and APP_{770} (*6–8*). APP_{695}, the mRNA that was initially identified (*5*), encodes an amyloid protein precursor (APP), 695 amino acids in length, that includes A4 at positions 597 to 638. APP_{751} is identical to APP_{695}, except for a 168-nucleotide insert (*6–8*). This insert, previously referred to as HL124i (*7*), would introduce 56 amino acids carboxyl terminal to Arg^{288} and convert Val^{289} into an isoleucine. APP_{770} is identical to APP_{751}, except for a 57-nucleo-

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M. L. Cohen and D. M. Kovacs, Division of Neuropathology, Institute of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH 44106.
R. E. Tanzi and J. F. Gusella, Neurogenetics Laboratory, Massachusetts General Hospital and Department of Genetics and the Program in Neuroscience, Harvard Medical School, Boston, MA 02115.

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L4 ANSWER 8 OF 8 BIOTECHDS COPYRIGHT 2005 THE THOMSON CORP. on STN
ACCESSION NUMBER: 1991-03225 BIOTECHDS
TITLE: Genetic disease detection and DNA amplification using cloned thermostable **ligase**;
ligase chain reaction using thermostable DNA-**ligase** of Thermus **aquaticus**
AUTHOR: Barany F
LOCATION: Department of Microbiology, Hearst Microbiology Research Center, Cornell University Medical College, 1300 York Avenue, New York, NY 10021, USA.
SOURCE: Proc.Natl.Acad.Sci.U.S.A.; (1991) 88, 1, 189-93
CODEN: PNASA6
DOCUMENT TYPE: Journal
LANGUAGE: English
AB A novel DNA detection system uses the thermostable DNA-**ligase** of Thermus **aquaticus** HB8 (ATCC 27634) to discriminate between a mismatched and complementary DNA helix. The enzyme specifically links 2 adjacent oligonucleotides when hybridized at 65 deg to a complementary target only when the nucleotides are perfectly base-paired at the junction. Because the enzyme retains activity after multiple thermal cycles, the ligations may be repeated to increase product (termed **ligase detection reaction**). Product is further amplified in a **ligase** chain reaction (LCR) by using both strands of genomic DNA as targets for oligonucleotide hybridization. 2 Sets of adjacent oligonucleotides, complementary to each target strand, are used. The ligation products from 1 round become the targets for the next round of ligation. By use of LCR, the amount of product can be increased exponentially by repeated thermal cycling. A single-base mismatch prevents ligation/amplification and is thus distinguished. The method was used to discriminate between normal beta-A and sickle beta-S globin genotypes from 10 ul blood samples. (30 ref)

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L4 ANSWER 1 OF 8 BIOTECHDS COPYRIGHT 2005 THE THOMSON CORP. on STN
ACCESSION NUMBER: 2005-04578 BIOTECHDS
TITLE: Identifying a target nucleic acid sequence variation comprises providing oligonucleotide probe sets, each comprising a target-specific portion and a barcode; DNA probe and DNA array for use in sequence variation identification
AUTHOR: DELGROSSO K; FORTINA P; GRAVES D; SURREY S
PATENT ASSIGNEE: UNIV JEFFERSON THOMAS; UNIV PENNSYLVANIA
PATENT INFO: WO 2005001113 6 Jan 2005
APPLICATION INFO: WO 2004-US20464 25 Jun 2004
PRIORITY INFO: US 2003-483352 27 Jun 2003; US 2003-483352 27 Jun 2003
DOCUMENT TYPE: Patent
LANGUAGE: English
OTHER SOURCE: WPI: 2005-075577 [08]
AB DERWENT ABSTRACT:

NOVELTY - Identifying a target nucleic acid sequence variation comprises providing one or more oligonucleotide probe sets, each set characterized by (i) a first oligonucleotide probe, having a first target-specific portion and a first barcode, and (ii) a second oligonucleotide probe, having a second target-specific portion and a second barcode.

DETAILED DESCRIPTION - Identifying a target nucleic acid sequence variation comprises: (a) in (M1), providing a sample potentially containing one or more target polynucleotide; (b) providing one or more oligonucleotide probe sets, each set characterized by: (i) a first oligonucleotide probe, having a first target-specific portion and a first barcode; and (ii) a second oligonucleotide probe, having a second target-specific portion and a second barcode, where the first oligonucleotide probe and the second oligonucleotide probe in a particular set are for ligation together when hybridized adjacent to one another on a corresponding target polynucleotide; (c) providing a **ligase**; (d) blending the sample, the one or more oligonucleotide probe sets, and the **ligase** to form a mixture; (e) subjecting the mixture to one or more **ligase detection reaction** cycles comprising a hybridization treatment, a ligation step and a denaturation treatment, where the oligonucleotide probe sets hybridize at adjacent positions to form a ligated product containing the first barcode, the target-specific portions connected together, and the second barcode; (f) providing a solid support with one or more surface-bound probes on an array, where the surface-bound probes are complementary to the first barcode; (g) contacting the ligated product of step (e) with the solid support under conditions for hybridization of the first barcode with the surface-bound probes; (h) providing a third barcode carrying one or more detectable labels and a nanoparticle attached into it, where the third barcode is complementary to the second barcode; and (i) detecting the presence of the detectable labels on the ligated product captured on the solid support at particular sites, thus detecting the nucleic acid sequence variation in the sample; or (j) in (M2), providing a sample potentially containing one or more target polynucleotides with at least one nucleotide variation; (k) providing one or more oligonucleotide probe sets, each set characterized by: (i) a first oligonucleotide probe, having a first target-specific portion and a first barcode; (ii) a second oligonucleotide probe, having a second target-specific portion and a second barcode; and (iii) a third oligonucleotide probe, having a third target-specific portion and a third barcode, where the first target-specific portion in the first oligonucleotide probe in a particular set is for ligation with the second target-specific portion in the second oligonucleotide probe, or the third target-specific portion in the third oligonucleotide probe; (l) providing a **ligase**; (m) blending the sample, the oligonucleotide probe

sets, and the **ligase** to form a mixture; (n) subjecting the mixture to one or more **ligase detection reaction** cycles comprising a hybridization treatment and a denaturation treatment, where the oligonucleotide probe sets hybridize at adjacent positions to form at least two ligated product, the first ligated product containing the first barcode, the first target-specific portion connected with the second target-specific portion, and the second barcode, the second ligated product containing the first barcode, the first target-specific portion connected with the third target-specific portion, and the third barcode; (o) providing a solid support with surface-bound probes on an array, where the surface-bound probes are complementary to the first barcode; (p) contacting the first and the second ligated product of step (n) with the solid support under conditions for hybridization of the first barcode with the surface-bound probes; (q) providing a fourth barcode carrying one or more detectable labels and a nanoparticle attached into it, where the fourth barcode is complementary to the second barcode; (r) providing a fifth barcode carrying one or more detectable labels and a nanoparticle attached into it, where the fifth barcode is complementary to the third barcode; and (s) detecting the presence of the detectable labels on the first ligated product, the second ligated product, or both on the solid support at a particular site, thus indicating the presence of one or more nucleic acid variation in a sample. An INDEPENDENT CLAIM is also included for a diagnostic test kit, for detecting nucleic acid variations in a sample, comprising (i) oligonucleotide probe sets, each set characterized by: (a) a first oligonucleotide probe, having a target-specific portion and a first barcode; (b) a second oligonucleotide probe, having a target-specific portion and a second barcode, where the first oligonucleotide probe and the second oligonucleotide probes in a particular set are for ligation together when hybridized adjacent to one another on a corresponding target polynucleotide, but have a mismatch which interferes with such ligation when hybridized to any other polynucleotide present in the sample; (c) **ligase** reagents; and (d) a third barcode carrying one or more detectable labels and a nanoparticle attached into it, where the third barcode is complementary to the second barcode.

BIOTECHNOLOGY - Preferred Method: The target nucleic acid sequence variation is a single nucleotide polymorphism. The surface-bound probes capture a normal target polynucleotide, a mutant target polynucleotide, or both, where the nanoparticle is attached at a 5' end or a 3' end of the third barcode. The detectable labels comprise one or more dyes, which have different surface-enhanced Raman spectra signatures, where dyes comprise cyanine dye, R110, R6, TAMRA, ROX, FAM, JOE, ZOE, TET, HEX, NAN, Texas Red, Rhodamine Red, Alexa dyes, or a combination, and where cyanine dye comprises CYA, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, or Cy7.5.3. The mutant target polynucleotide differs from the target polynucleotide in the sample at one or more single nucleotide positions. The nucleic acid sequence variations comprise multiple allele differences at a single nucleotide position, at two or more nucleotide positions, or at nucleotide positions in multiple target polynucleotides. The target-specific portions of the oligonucleotide probe sets have substantially the same melting temperature so that they hybridize to the target polynucleotides under similar hybridization conditions. The nucleic acid sequence variations comprise insertions, deletions, microsatellite repeats, translocations, mutations, or a combination. The denaturation treatment is at 70-105degreesC. The target-specific portions of the oligonucleotide probes each have a hybridization temperature of 40-85degreesC, preferably 60-70degreesC. The denaturation and the hybridization are 30 seconds to 5 minutes long. Step (e), of (M1), is repeated for 2-50 cycles and takes 1-250 minutes. The **ligase** is Thermus **aquaticus ligase**, Thermus thermophilus **ligase**, Escherichia coli **ligase**, T4 DNA **ligase** , Thermus sp., AK166 **ligase**, Aquifex aeolicus **ligase**,

Thermotoga maritima **ligase**, and Pyrococcus **ligase**. The target-specific portions of the oligonucleotide probes are 15-30 nucleotides long. The method further comprises amplifying the target polynucleotides in the sample prior to the **ligase**, where amplification is carried out by subjecting the sample to a polymerase-based amplifying procedure. The solid support is made from a material, e.g. plastic, ceramic, metal, resin, gel, glass, silicon, and their composites. The method further comprises treating the ligated product chemically or enzymatically after step (e), of (M1) to remove unligated oligonucleotide probes, where the treatment is carried out with an exonuclease. The target polynucleotide is a genomic DNA. The ligated product is amplified with additional universal primers and DNA polymerase after ligation. The second and the third oligonucleotide probes capture allelic variants of a target polynucleotide. The detectable label carried by the fourth barcode comprises a first dye and the detectable label carried by the fifth barcode comprises a second dye, where the first and second dyes have different surface-enhanced Raman spectra signatures. Preferred Diagnostic Test Kit: The third barcode comprises at least two different barcode sequences. The nanoparticle and the third barcode are in separate containers, and the third barcode is attached to the nanoparticles prior to performing an assay. The nanoparticle, the third barcode, or both are functionalized prior to attachment of the nanoparticle. The diagnostic test kit further contains a substrate, the substrate having attached into it a probe that hybridizes to the first barcode, where the probe is a DNA microarray.

USE - The methods and kit are useful for identifying a target nucleic acid sequence variation. (53 pages)

L4 ANSWER 2 OF 8 CAPLUS COPYRIGHT 2005 ACS on STN
ACCESSION NUMBER: 2001:781173 CAPLUS
DOCUMENT NUMBER: 135:340147
TITLE: Probe sequence requirements and the design of addressable arrays for detection of sequence differences using ligase detection reaction
INVENTOR(S): Barany, Francis; Zirvi, Monib; Gerry, Norman P.; Favis, Reyna; Kliman, Richard
PATENT ASSIGNEE(S): Cornell Research Foundation, Inc., USA
SOURCE: PCT Int. Appl., 300 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 2
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.		DATE
WO 2001079548	A2	20011025	WO 2001-US10958		20010404
WO 2001079548	A3	20030206			
W: AU, CA, JP, US					
RW: AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR					
CA 2405412	AA	20011025	CA 2001-2405412		20010404
WO 2001079548	A2	20011025	WO 2001-XA10958		20010404
W: AU, CA, JP, US					
RW: AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR					
EP 1303639	A2	20030423	EP 2001-969050		20010404
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI, CY, TR					
JP 2004526402	T2	20040902	JP 2001-577530		20010404
PRIORITY APPLN. INFO.:			US 2000-197271P	P	20000414
			WO 2001-US10958	W	20010404

AB The present invention is directed to a method of designing a plurality of

capture oligonucleotide probes for use on a support to which complementary oligonucleotide probes will hybridize with little mismatch, where the plural capture oligonucleotide probes have melting temps. within a narrow range. The first step of the method involves providing a first set of a plurality of tetramers of four nucleotides linked together, where (1) each tetramer within the set differs from all other tetramers in the set by at least two nucleotide bases, (2) no two tetramers within a set are complementary to one another, (3) no tetramers within a set are palindromic or dinucleotide repeats, and (4) no tetramer within a set has one or less or three or more G or C nucleotides. Groups of 2 to 4 of the tetramers from the first set are linked together to form a collection of multimer units. From the collection of multimer units, all multimer units formed from the same tetramer and all multimer units having a melting temperature in °C of less than 4 times the number of tetramers forming a multimer unit are removed to form a modified collection of multimer units. The modified collection of multimer units is arranged in a list in order of melting temperature The order of the modified collection of multimer units is randomized in 2°C increments of melting temperature

L4 ANSWER 3 OF 8 CAPLUS COPYRIGHT 2005 ACS on STN
ACCESSION NUMBER: 2000:688408 CAPLUS
DOCUMENT NUMBER: 133:262262
TITLE: Detection of nucleic acid polymorphisms using the **ligase detection reaction** with addressable arrays of capture probes
INVENTOR(S): Barany, Francis; Gerry, Norman P.; Witowski, Nancy E.; Day, Joseph; Hammer, Robert P.; Barany, George
PATENT ASSIGNEE(S): Cornell Research Foundation, Inc., USA; Regents of the University of Minnesota; Board of Supervisors of Louisiana State University and Agricultural and Mechanical College
SOURCE: PCT Int. Appl., 217 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2000056927	A2	20000928	WO 2000-US7006	20000317
WO 2000056927	A3	20020314		
W: AU, CA, JP				
RW: AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE				
US 6506594	B1	20030114	US 2000-526992	20000316
CA 2366249	AA	20000928	CA 2000-2366249	20000317
EP 1208223	A2	20020529	EP 2000-916438	20000317
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI, CY				
JP 2003520570	T2	20030708	JP 2000-606786	20000317
US 2003175750	A1	20030918	US 2002-272152	20021015
PRIORITY APPLN. INFO.:			US 1999-125357P P	19990319
			US 2000-526992 A3	20000316
			WO 2000-US7006 W	20000317

AB The present invention describes a method for identifying one or more of a plurality of sequences differing by one or more single base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. The ligation phase utilizes a ligation **detection reaction** between one oligonucleotide probe which has a target sequence-specific portion and an addressable array-specific portion, and a second oligonucleotide probe, having a target sequence specific portion and a detectable label. After the

ligation phase, the capture phase is carried out by hybridizing the ligated oligonucleotide probes to a solid support with an array of immobilized capture oligonucleotides at least some of which are complementary to the addressable array-specific portion. Following completion of the capture phase, a detection phase is carried out to detect the labels of ligated oligonucleotide probes hybridized to the solid support. The ligation phase can be preceded by an amplification process. The present invention also relates to a kit for practicing this method, a method of forming arrays on solid supports, and the supports themselves.

L4 ANSWER 4 OF 8 CAPLUS COPYRIGHT 2005 ACS on STN DUPLICATE 1
ACCESSION NUMBER: 1997:579861 CAPLUS
DOCUMENT NUMBER: 127:215947
TITLE: Detection of nucleic acid sequence differences using the **ligase detection reaction** with addressable array
INVENTOR(S): Barany, Francis; Barany, George; Hammer, Robert P.; Kempe, Maria; Blok, Herman; Zirvi, Monib
PATENT ASSIGNEE(S): Cornell Research Foundation, Inc., USA; University of Minnesota; Louisiana State University; Barany, Francis; Barany, George; Hammer, Robert P.; Kempe, Maria; Blok, Herman; Zirvi, Monib
SOURCE: PCT Int. Appl., 124 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 2
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.		DATE
WO 9731256	A2	19970828	WO 1997-US1535		19970205
W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM					
RW: KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG					
CA 2244891	AA	19970828	CA 1997-2244891		19970205
AU 9727997	A1	19970910	AU 1997-27997		19970205
AU 735440	B2	20010705			
EP 920440	A2	19990609	EP 1997-922283		19970205
R: CH, DE, FR, GB, IT, LI, SE					
JP 2001519648	T2	20011023	JP 1997-530164		19970205
PRIORITY APPLN. INFO.:			US 1996-11359P	P	19960209
			WO 1997-US1535	W	19970205

AB The present invention describes a method for identifying one or more of a plurality of sequences differing by one or more single base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. The method includes a ligation phase, a capture phase, and a detection phase. The ligation phase utilizes a ligation **detection reaction** between one oligonucleotide probe, which has a target sequence-specific portion and an addressable array-specific portion, and a second oligonucleotide probe, having a target sequence-specific portion and a detectable label. After the ligation phase, the capture phase is carried out by hybridizing the ligated oligonucleotide probes to a solid support with an array of immobilized capture oligonucleotides at least some of which are complementary to the addressable array-specific portion. Following completion of the capture phase, a detection phase is carried out to

detect the labels of ligated oligonucleotide probes hybridized to the solid support. The ligation phase can be preceded by an amplification process. The present invention also relates to a kit for practicing this method, a method of forming arrays on solid supports, and the supports themselves.

L4 ANSWER 5 OF 8 CAPLUS COPYRIGHT 2005 ACS on STN
ACCESSION NUMBER: 1997:805853 CAPLUS
DOCUMENT NUMBER: 128:58283
TITLE: Detection of nucleic acid sequence differences using coupled **ligase** detection and polymerase chain reactions
INVENTOR(S): Belgrader, Phillip; Barany, Francis; Lubin, Matthew
PATENT ASSIGNEE(S): Cornell Research Foundation, Inc., USA; Belgrader, Phillip
SOURCE: PCT Int. Appl., 158 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9745559	A1	19971204	WO 1997-US9012	19970527
W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
CA 2255774	AA	19971204	CA 1997-2255774	19970527
AU 9732160	A1	19980105	AU 1997-32160	19970527
AU 730633	B2	20010308		
EP 912761	A1	19990506	EP 1997-927787	19970527
R: CH, DE, FR, GB, IT, LI, SE				
JP 2000511060	T2	20000829	JP 1997-542878	19970527
US 6027889	A	20000222	US 1997-864473	19970528
US 6268148	B1	20010731	US 1999-440523	19991115
US 2003032016	A1	20030213	US 2001-918156	20010730
US 6797470	B2	20040928		
US 2004203061	A1	20041014	US 2004-843720	20040512
US 2004214224	A1	20041028	US 2004-852289	20040524

PRIORITY APPLN. INFO.:

US 1996-18532P	P	19960529
WO 1997-US9012	W	19970527
US 1997-864473	A3	19970528
US 1999-440523	A1	19991115
US 2001-918156	A1	20010730

AB The present invention relates to the detection of nucleic acid sequence differences using coupled **ligase detection reaction** (**LDR**) and polymerase chain reaction (PCR). One aspect of the present invention involves use of a **ligase detection reaction** coupled to a polymerase chain reaction. Another aspect of the present invention relates to the use of a primary polymerase chain reaction coupled to a secondary polymerase chain reaction coupled to a **ligase detection reaction**. A third aspect of the present invention involves a primary polymerase chain reaction coupled to a secondary polymerase chain reaction. Such coupling of the **ligase detection reaction** and the polymerase chain reaction permits multiplex detection of nucleic acid sequence differences. Several rapid, single

assay formats are presented to detect the presence or absence of multiple selected sequence in a polynucleotide same that differ by single-base changes, insertions, deletions, translocations, and/or allele differences. Each of these embodiments have particular applicability in detecting certain characteristics, but possess the common characteristic that each requires the use of coupled reactions for multiplex detection of nucleic acid sequences differences where oligonucleotides from an early phase of each process contain sequences which may be used by oligonucleotides from a later phase of the process.

L4 ANSWER 6 OF 8 BIOTECHDS COPYRIGHT 2005 THE THOMSON CORP. on STN
ACCESSION NUMBER: 1994-00870 BIOTECHDS
TITLE: Method for detecting mutated gene or oncogene in sample; e.g. k-ras gene mutation detection by polymerase chain reaction, allele-specific **ligase** chain reaction or **ligase detection reaction** , for e.g. tumor diagnosis
PATENT ASSIGNEE: Dartmouth-Coll.
PATENT INFO: WO 9322456 11 Nov 1993
APPLICATION INFO: WO 1993-US3561 14 Apr 1993
PRIORITY INFO: US 1992-874845 27 Apr 1992
DOCUMENT TYPE: Patent
LANGUAGE: English
OTHER SOURCE: WPI: 1993-368814 [46]
AB A new method for detecting a mutant allele comprises: extracting DNA from a biological fluid sample (whole blood, serum, plasma, urine, sputum or cerebrospinal fluid); denaturing the DNA to form 2 strands; amplifying the mutant allele using at least 1 set of 4 allele-specific oligonucleotide primers, containing 1 primer complementary to a mutation-containing segment on the 1st strand, and a 1st common primer for pairing during amplification to each allele-specific pair, complementary to a segment of the 2nd strand of DNA; and detecting the mutant allele. Amplification may be by the polymerase chain reaction (using Thermus **aquaticus** Taq DNA-polymerase (EC-2.7.7.7) lacking 3'-exonuclease activity), allele-specific **ligase** chain reaction or **ligase detection reaction**. The mutant allele is the k-ras gene with a mutation at position 1 or 2 in the 12th codon. The method is useful e.g. in cancer diagnosis. (49pp)

L4 ANSWER 7 OF 8 CAPLUS COPYRIGHT 2005 ACS on STN
ACCESSION NUMBER: 1992:122626 CAPLUS
DOCUMENT NUMBER: 116:122626
TITLE: A thermostable DNA **ligase** for use in diagnostic nucleic acid amplification
INVENTOR(S): Barany, Francis; Zebala, John; Nickerson, Deborah A.; Kaiser, Robert J., Jr.; Hood, Leroy
PATENT ASSIGNEE(S): Cornell Research Foundation, Inc., USA; California Institute of Technology
SOURCE: PCT Int. Appl.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9117239	A1	19911114	WO 1991-US2968	19910429
W: JP, US				
RW: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, NL, SE				
EP 528882	A1	19930303	EP 1991-909119	19910429
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE				
JP 05508764	T2	19931209	JP 1991-508891	19910429

JP 2001269187	A2	20011002	JP 2001-60432	19910429
EP 1507000	A2	20050216	EP 2004-77667	19910429
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE				
CA 2067991	AA	19931106	CA 1992-2067991	19920505
US 5494810	A	19960227	US 1994-343785	19941122
US 5830711	A	19981103	US 1995-462221	19950605
US 6054564	A	20000425	US 1997-946458	19971007
US 2004048308	A1	20040311	US 2003-662199	20030912
JP 2004089204	A2	20040325	JP 2003-398813	20031128
PRIORITY APPLN. INFO.:			US 1990-518447	A 19900503
			EP 1991-909119	A3 19910429
			JP 1991-508891	A3 19910429
			WO 1991-US2968	W 19910429
			US 1992-971095	B1 19921102
			US 1994-343785	A1 19941122
			US 1995-462221	A3 19950605
			US 1997-946458	A3 19971007
			US 2000-480515	A1 20000110

AB **Ligase** chain reaction and **ligase detection reaction** are made more efficient and the noise level reduced by using higher temps. and a thermostable DNA **ligase** from Thermus **aquaticus**. The gene for the T. **aquaticus ligase** is cloned and expressed in Escherichia coli. The gene was cloned by complementation in an E. coli with a temperature-sensitive **ligase** and the gene was placed under control of a T7 promoter or the phoA promoter for high level expression. Chromatog. purification of the protein from lysates of cells in which the gene was under control of the phoA promoter yielded 6 mg of enzyme (107 units) from 2 L of culture (.apprx.105-fold purification). When used at 65° the enzyme was capable of catalyzing the **ligase detection reaction** and the **ligase** chain reaction. Control studies indicated that the enzyme was inactive against mismatched combinations of primer and target but was active when the match was perfect. The enzyme was active with femtomolar substrate concns. The use of the enzyme in the detection of β-globin alleles was demonstrated.

L4 ANSWER 8 OF 8 BIOTECHDS COPYRIGHT 2005 THE THOMSON CORP. on STN
ACCESSION NUMBER: 1991-03225 BIOTECHDS
TITLE: Genetic disease detection and DNA amplification using cloned thermostable **ligase**;
ligase chain reaction using thermostable DNA-**ligase** of Thermus **aquaticus**
AUTHOR: Barany F
LOCATION: Department of Microbiology, Hearst Microbiology Research Center, Cornell University Medical College, 1300 York Avenue, New York, NY 10021, USA.
SOURCE: Proc.Natl.Acad.Sci.U.S.A.; (1991) 88, 1, 189-93
CODEN: PNASA6
DOCUMENT TYPE: Journal
LANGUAGE: English

AB A novel DNA detection system uses the thermostable DNA-**ligase** of Thermus **aquaticus** HB8 (ATCC 27634) to discriminate between a mismatched and complementary DNA helix. The enzyme specifically links 2 adjacent oligonucleotides when hybridized at 65 deg to a complementary target only when the nucleotides are perfectly base-paired at the junction. Because the enzyme retains activity after multiple thermal cycles, the ligations may be repeated to increase product (termed **ligase detection reaction**). Product is further amplified in a **ligase** chain reaction (LCR) by using both strands of genomic DNA as targets for oligonucleotide hybridization. 2 Sets of adjacent oligonucleotides, complementary to each target strand, are used. The ligation products from 1 round become the targets for the next round of ligation. By use of LCR, the amount of product can be

increased exponentially by repeated thermal cycling. A single-base mismatch prevents ligation/amplification and is thus distinguished. The method was used to discriminate between normal beta-A and sickle beta-S globin genotypes from 10 ul blood samples. (30 ref)

Refine Search

Search Results -

Terms	Documents
L2 same detection	32

Database: US Pre-Grant Publication Full-Text Database; US Patents Full-Text Database; US OCR Full-Text Database; EPO Abstracts Database; JPO Abstracts Database; Derwent World Patents Index; IBM Technical Disclosure Bulletins

Search: L3

Refine Search | Recall Text | Clear | Interrupt

Search History

DATE: Wednesday, April 20, 2005 Printable Copy Create Case

Set Name side by side	Query	Hit Count	Set Name result set
DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI; PLUR=YES; OP=ADJ			
L3	L2 same detection	32	L3
L2	L1 same thermo$	609	L2
L1	ligase	30020	L1

END OF SEARCH HISTORY

Hit List

Clear | Generate Collection | Print | Fwd Refs | Bkwd Refs | Generate OACS

Search Results - Record(s) 1 through 32 of 32 returned.

☐ 1. Document ID: US 20050064459 A1

Using default format because multiple data bases are involved.

L3: Entry 1 of 32 File: PGPB Mar 24, 2005

PGPUB-DOCUMENT-NUMBER: 20050064459
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20050064459 A1

TITLE: Ligation assay

PUBLICATION-DATE: March 24, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Lao, Kai Qin	Pleasanton	CA	US	

US-CL-CURRENT: 435/6

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 2. Document ID: US 20050037398 A1

L3: Entry 2 of 32 File: PGPB Feb 17, 2005

PGPUB-DOCUMENT-NUMBER: 20050037398
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20050037398 A1

TITLE: 2'-terminator nucleotide-related methods and systems

PUBLICATION-DATE: February 17, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Gelfand, David Harrow	Oakland	CA	US	
Reichert, Fred Lawrence	San Leandro	CA	US	
Bodepudi, Veeraiah	San Ramon	CA	US	
Gupta, Amar	Danville	CA	US	
Will, Stephen	Oakland	CA	US	
Myers, Thomas	Alameda	CA	US	

US-CL-CURRENT: 435/6; 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 3. Document ID: US 20040137484 A1

L3: Entry 3 of 32 File: PGPB Jul 15, 2004

PGPUB-DOCUMENT-NUMBER: 20040137484
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040137484 A1

TITLE: Nucleic acid amplification methods

PUBLICATION-DATE: July 15, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Zhang, David Y.	Jamaica	NY	US	
Zhang, Wandi	New York	NY	US	
Yi, Jizu	Bayside	NY	US	

US-CL-CURRENT: 435/6; 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 4. Document ID: US 20040106112 A1

L3: Entry 4 of 32 File: PGPB Jun 3, 2004

PGPUB-DOCUMENT-NUMBER: 20040106112
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040106112 A1

TITLE: Nucleic acid detection medium

PUBLICATION-DATE: June 3, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Nilsson, Mats Bo Johan	Uppsala		SE	
Landegren, Ulf	Uppsala		SE	

US-CL-CURRENT: 435/6; 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 5. Document ID: US 20040077587 A1

L3: Entry 5 of 32 File: PGPB Apr 22, 2004

PGPUB-DOCUMENT-NUMBER: 20040077587
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040077587 A1

TITLE: 2'-C-methyl-3'-O-L-valine ester ribofuranosyl cytidine for treatment of flaviviridae infections

PUBLICATION-DATE: April 22, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Sommadossi, Jean-Pierre	Cambridge	MA	US	
LaColla, Paola	Cagliari		IT	

US-CL-CURRENT: 514/50; 536/28.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 6. Document ID: US 20030207300 A1

L3: Entry 6 of 32 File: PGPB Nov 6, 2003

PGPUB-DOCUMENT-NUMBER: 20030207300
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030207300 A1

TITLE: Multiplex analytical platform using molecular tags

PUBLICATION-DATE: November 6, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Matray, Tracy J.	Campbell	CA	US	
Singh, Sharat S.	San Jose	CA	US	
Macevicz, Stephen C.	Cupertino	CA	US	

US-CL-CURRENT: 435/6; 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 7. Document ID: US 20030190604 A1

L3: Entry 7 of 32 File: PGPB Oct 9, 2003

PGPUB-DOCUMENT-NUMBER: 20030190604
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030190604 A1

TITLE: Nucleic acid amplification method: ramification-extension amplification method (RAM)

PUBLICATION-DATE: October 9, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Zhang, David Y.	Jamaica	NY	US	
Brandwein, Margaret	Jamaica Estates	NY	US	
Hsuih, Terence C.H.	Long Island City	NY	US	

US-CL-CURRENT: 435/5; 435/6, 435/91.2

☐ 8. Document ID: US 20030175706 A1

L3: Entry 8 of 32 File: PGPB Sep 18, 2003

PGPUB-DOCUMENT-NUMBER: 20030175706
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030175706 A1

TITLE: Nucleic acid amplification methods

PUBLICATION-DATE: September 18, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Zhang, David Y.	Jamaica	NY	US	

US-CL-CURRENT: 435/6; 435/91.2

☐ 9. Document ID: US 20030143600 A1

L3: Entry 9 of 32 File: PGPB Jul 31, 2003

PGPUB-DOCUMENT-NUMBER: 20030143600
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030143600 A1

TITLE: Detection of extracellular tumor-associated nucleic acid in blood plasma or serum using nucleic acid amplification assays

PUBLICATION-DATE: July 31, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Gocke, Christopher D.	Ellicott City	MD	US	
Kopreski, Michael S.	Long Valley	NJ	US	
Benko, Floyd A.	Palmyra	PA	US	

US-CL-CURRENT: 435/6; 435/91.2

☐ 10. Document ID: US 20030096258 A1

L3: Entry 10 of 32 File: PGPB May 22, 2003

PGPUB-DOCUMENT-NUMBER: 20030096258
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030096258 A1

TITLE: Solid phase sequencing of double-stranded nucleic acids

PUBLICATION-DATE: May 22, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Fu, Dong-Jing	Waltham	MA	US	
Cantor, Charles R.	Boston	MA	US	
Koster, Hubert	Concord	MA	US	
Smith, Cassandra L.	Boston	MA	US	

US-CL-CURRENT: 435/6; 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 11. Document ID: US 20020182598 A1

L3: Entry 11 of 32 File: PGPB Dec 5, 2002

PGPUB-DOCUMENT-NUMBER: 20020182598
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020182598 A1

TITLE: Nucleic acid amplification methods

PUBLICATION-DATE: December 5, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Zhang, David Y.	Jamaica	NY	US	

US-CL-CURRENT: 435/6; 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 12. Document ID: US 20020058270 A1

L3: Entry 12 of 32 File: PGPB May 16, 2002

PGPUB-DOCUMENT-NUMBER: 20020058270
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020058270 A1

TITLE: Methods and compositions for transcription-based nucleic acid amplification

PUBLICATION-DATE: May 16, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Kurn, Nurith	Palo Alto	CA	US	

US-CL-CURRENT: 435/6; 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 13. Document ID: US 6855523 B2

L3: Entry 13 of 32 File: USPT Feb 15, 2005

US-PAT-NO: 6855523
DOCUMENT-IDENTIFIER: US 6855523 B2

TITLE: Nucleic acid amplification method: ramification-extension amplification method (RAM)

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 14. Document ID: US 6821771 B2

L3: Entry 14 of 32 File: USPT Nov 23, 2004

US-PAT-NO: 6821771
DOCUMENT-IDENTIFIER: US 6821771 B2

TITLE: Device for thermo-dependent chain reaction amplification of target nucleic acid sequences, measured in real-time

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 15. Document ID: US RE38442 E

L3: Entry 15 of 32 File: USPT Feb 24, 2004

US-PAT-NO: RE38442
DOCUMENT-IDENTIFIER: US RE38442 E

TITLE: Nucleic acid amplification method hybridization signal amplification method (HSAM)

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 16. Document ID: US 6593120 B1

L3: Entry 16 of 32 File: USPT Jul 15, 2003

US-PAT-NO: 6593120
DOCUMENT-IDENTIFIER: US 6593120 B1
** See image for Certificate of Correction **

TITLE: Recombinant DNA encoding a reverse transcriptase derived from moloney murine leukemia virus

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 17. Document ID: US 6593086 B2

L3: Entry 17 of 32 File: USPT Jul 15, 2003

US-PAT-NO: 6593086
DOCUMENT-IDENTIFIER: US 6593086 B2

TITLE: Nucleic acid amplification methods

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 18. Document ID: US 6569647 B1

L3: Entry 18 of 32 File: USPT May 27, 2003

US-PAT-NO: 6569647
DOCUMENT-IDENTIFIER: US 6569647 B1

TITLE: Nucleic acid amplification method: ramification-extension amplification method (RAM)

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 19. Document ID: US 6569618 B1

L3: Entry 19 of 32 File: USPT May 27, 2003

US-PAT-NO: 6569618
DOCUMENT-IDENTIFIER: US 6569618 B1

TITLE: Diagnosis of diseases associated with coronary twitching

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 20. Document ID: US 6521409 B1

L3: Entry 20 of 32 File: USPT Feb 18, 2003

US-PAT-NO: 6521409
DOCUMENT-IDENTIFIER: US 6521409 B1

TITLE: Detection of extracellular tumor-associated nucleic acid in blood plasma or serum using nucleic acid amplification assays

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 21. Document ID: US 6451563 B1

L3: Entry 21 of 32 File: USPT Sep 17, 2002

US-PAT-NO: 6451563
DOCUMENT-IDENTIFIER: US 6451563 B1

TITLE: Method for making linear, covalently closed DNA constructs

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 22. Document ID: US 6436635 B1

L3: Entry 22 of 32 File: USPT Aug 20, 2002

US-PAT-NO: 6436635
DOCUMENT-IDENTIFIER: US 6436635 B1
**** See image for Certificate of Correction ****

TITLE: Solid phase sequencing of double-stranded nucleic acids

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 23. Document ID: US 6210897 B1

L3: Entry 23 of 32 File: USPT Apr 3, 2001

US-PAT-NO: 6210897
DOCUMENT-IDENTIFIER: US 6210897 B1

TITLE: Identification of canine leukocyte adhesion deficiency in dogs

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 24. Document ID: US 6156504 A

L3: Entry 24 of 32 File: USPT Dec 5, 2000

US-PAT-NO: 6156504
DOCUMENT-IDENTIFIER: US 6156504 A

TITLE: Detection of extracellular tumor-associated nucleic acid in blood plasma or serum using nucleic acid amplification assays

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 25. Document ID: US 6130073 A

L3: Entry 25 of 32 File: USPT Oct 10, 2000

US-PAT-NO: 6130073
DOCUMENT-IDENTIFIER: US 6130073 A

TITLE: Coupled amplification and ligation method

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 26. Document ID: US 5942391 A

L3: Entry 26 of 32 File: USPT Aug 24, 1999

US-PAT-NO: 5942391
DOCUMENT-IDENTIFIER: US 5942391 A
**** See image for Certificate of Correction ****

TITLE: Nucleic acid amplification method: ramification-extension amplification method (RAM)

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 27. Document ID: US 5912148 A

L3: Entry 27 of 32 File: USPT Jun 15, 1999

US-PAT-NO: 5912148
DOCUMENT-IDENTIFIER: US 5912148 A

TITLE: Coupled amplification and ligation method

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 28. Document ID: US 5876924 A

L3: Entry 28 of 32 File: USPT Mar 2, 1999

US-PAT-NO: 5876924
DOCUMENT-IDENTIFIER: US 5876924 A

TITLE: Nucleic acid amplification method hybridization signal amplification method (HSAM)

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 29. Document ID: US 5753439 A

L3: Entry 29 of 32 File: USPT May 19, 1998

US-PAT-NO: 5753439
DOCUMENT-IDENTIFIER: US 5753439 A

TITLE: Nucleic acid detection methods

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 30. Document ID: US 5516663 A

L3: Entry 30 of 32 File: USPT May 14, 1996

US-PAT-NO: 5516663
DOCUMENT-IDENTIFIER: US 5516663 A

TITLE: Ligase chain reaction with endonuclease IV correction and contamination

control

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 31. Document ID: US 5512430 A

L3: Entry 31 of 32 File: USPT Apr 30, 1996

US-PAT-NO: 5512430
DOCUMENT-IDENTIFIER: US 5512430 A
** See image for Certificate of Correction **

TITLE: Diagnostic array for virus infection

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 32. Document ID: US 20040241716 A1, WO 2004072238 A2

L3: Entry 32 of 32 File: DWPI Dec 2, 2004

DERWENT-ACC-NO: 2004-635194
DERWENT-WEEK: 200481

TITLE: New terminal-phosphate labeled nucleoside polyphosphate useful for increasing rate of enzyme catalyzed nucleoside monophosphate transfer or determining identity of single nucleotide in nucleic acid sequence

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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L2 same detection	32

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Gene
Volume 76, Issue 2 , 30 March 1989, Pages 245-254

doi:10.1016/0378-1119(89)90165-0 Cite or Link Using DOI

Specificity of the nick-closing activity of bacteriophage T4 DNA ligase

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Received 16 September 1988; revised 25 October 1988; accepted 26 October 1988.; Available online 16 January 2003.

Abstract

Bacteriophage T4 DNA ligase effectively joins two adjacent, short synthetic oligodeoxyribonucleotides (oligos), as guided by complementary oligo, plasmid and genomic DNA templates. When a single bp mismatch exists at either side of the ligation junction, the efficiency of the enzyme to ligate the two oligos decreases. Mismatch ligation is approximately five-fold greater if the mismatch occurs at the 3' side rather than at the 5' side of the junction. During mismatch ligation the 5' adenylate of the 3' oligo accumulates in the reaction. The level of the adenylate formation correlates closely with the level of the mismatch ligation. Both mismatch ligation and adenylate formation are suppressed at elevated temperatures and in the presence of 200 mM NaCl or 2–5 mM spermidine. The apparent K_m for the oligo template in the absence of salt is 0.05 μM, whereas the K_m increases to 0.2 μM in the presence of 200 mM of NaCl. In this report, we demonstrate these properties of T4 DNA ligase for oligo pairs complementary to the β-globin gene at the sequence surrounding the single bp mutation responsible for sickle-cell anemia. Because of the highly specific nature of the nick-closing reaction, ligation of short oligos with DNA ligase can be used to distinguish two DNA templates differing by a single nucleotide.

Author Keywords: Recombinant DNA; genetic diseases; nucleic acid modifying enzymes; oligodeoxyribonucleotide

Abbreviations: bp, base pair(s); CIAP, calf intestinal alkaline phosphatase; DTT, dithiothreitol; EBV, Epstein-Barr virus; Exo III, exonuclease III; Hβ19A, Hβ19S, Hβ23A'; Hβ23S', see Table I; HPLC, high-performance liquid chromatography; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ON1, ONA2, ONS2, ONA3, ONS3 and ON4, see Fig. 1

and Table I; PEG, polyethylene glycol; PP_i, inorganic pyrophosphate; TBE, 89 mM Tris/89 mM boric acid/2 mM EDTA

Gene
Volume 76, Issue 2 , 30 March 1989, Pages 245-254